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(54) Title: CATIONIC AMPHIPHILES AND PLASMIDS FOR INTRACELLULAR DELIVERY OF THERAPEUTIC MOLECULES			
(57) Abstract			
<p>Novel cationic amphiphiles are provided that facilitate transport of biologically active (therapeutic) molecules into cells. The amphiphiles contain lipophilic groups derived from steroids, from mono or dialkylamines, or from alkyl or acyl groups; and cationic groups, protonatable at physiological pH, derived from amines, alkylamines or polyalkylamines. There are provided also therapeutic compositions prepared typically by contacting a dispersion of one or more cationic amphiphiles with the therapeutic molecules. Therapeutic molecules that can be delivered into cells according to the practice of the invention include DNA, RNA, and polypeptides. Representative uses of the therapeutic compositions of the invention include providing gene therapy, and delivery of antisense polynucleotides or biologically active polypeptides to cells. With respect to therapeutic compositions for gene therapy, the DNA is provided typically in the form of a plasmid for complexing with the cationic amphiphile. Novel and highly effective plasmid constructs are also disclosed, including those that are particularly effective at providing gene therapy for clinical conditions complicated by inflammation. Additionally, targeting of organs for gene therapy by intravenous administration of therapeutic compositions is described.</p>			

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**Cationic Amphiphiles and Plasmids for  
Intracellular Delivery of Therapeutic Molecules**

5

**Background of the Invention**

The present invention relates to novel cationic amphiphilic compounds that facilitate the intracellular delivery of biologically active (therapeutic) molecules. The present invention relates also to pharmaceutical compositions that comprise such cationic amphiphiles, and  
10 that are useful to deliver into the cells of patients therapeutically effective amounts of biologically active molecules. The novel cationic amphiphilic compounds of the invention are particularly useful in relation to gene therapy.

Effective therapeutic use of many types of biologically active  
15 molecules has not been achieved simply because methods are not available to cause delivery of therapeutically effective amounts of such substances into the particular cells of a patient for which treatment therewith would provide therapeutic benefit. Efficient delivery of therapeutically sufficient amounts of such molecules into cells has often proved difficult, if not  
20 impossible, since, for example, the cell membrane presents a selectively-permeable barrier. Additionally, even when biologically active molecules successfully enter targeted cells, they may be degraded directly in the cell cytoplasm or even transported to structures in the the cell, such as lysosomal compartments, specialized for degradative processes. Thus both  
25 the nature of substances that are allowed to enter cells, and the amounts thereof that ultimately arrive at targeted locations within cells, at which they can provide therapeutic benefit, are strictly limited.

Although such selectivity is generally necessary in order that proper cell function can be maintained, it comes with the disadvantage that many therapeutically valuable substances (or therapeutically effective amounts thereof) are excluded. Additionally, the complex structure, behavior, and environment presented by an intact tissue that is targeted for intracellular delivery of biologically active molecules often interfere substantially with such delivery, in comparison with the case presented by populations of cells cultured *in vitro*.

Examples of biologically active molecules for which effective targeting to a patients' tissues is often not achieved: (1) numerous proteins including immunoglobulin proteins, (2) polynucleotides such as genomic DNA, cDNA, or mRNA (3) antisense polynucleotides; and (4) many low molecular weight compounds, whether synthetic or naturally occurring, such as the peptide hormones and antibiotics.

One of the fundamental challenges now facing medical practitioners is that although the defective genes that are associated with numerous inherited diseases (or that represent disease risk factors including for various cancers) have been isolated and characterized, methods to correct the disease states themselves by providing patients with normal copies of such genes (the technique of gene therapy) are substantially lacking. Accordingly, the development of improved methods of intracellular delivery therefor is of great medical importance. Examples of diseases that it is hoped can be treated by gene therapy include inherited disorders such as cystic fibrosis, Gaucher's disease, Fabry's disease, and muscular dystrophy. Representative of acquired disorders that can be treated are: (1) for cancers — multiple myeloma, leukemias, melanomas, ovarian carcinoma and small cell lung cancer; (2) for cardiovascular conditions — progressive

heart failure, restenosis, and hemophilias; and (3) for neurological conditions — traumatic brain injury.

Gene therapy requires successful transfection of target cells in a patient. Transfection may generally be defined as the process of introducing  
5 an expressible polynucleotide (for example a gene, a cDNA, or an mRNA patterned thereon) into a cell. Successful expression of the encoding polynucleotide leads to production in the cells of a normal protein and leads to correction of the disease state associated with the abnormal gene. Therapies based on providing such proteins directly to target cells (protein  
10 replacement therapy) are often ineffective for the reasons mentioned above.

Cystic fibrosis, a common lethal genetic disorder, is a particular example of a disease that is a target for gene therapy. The disease is caused by the presence of one or more mutations in the gene that encodes a protein known as cystic fibrosis transmembrane conductance regulator ("CFTR"),  
15 and which regulates the movement of ions (and therefore fluid) across the cell membrane of epithelial cells, including lung epithelial cells. Abnormal ion transport in airway cells leads to abnormal mucous secretion, inflammation and infection, tissue damage, and eventually death.

It is widely hoped that gene therapy will provide a long lasting and  
20 predictable form of therapy for certain disease states, and it is likely the only form of therapy suitable for many inherited diseases. There remains however a critical need to develop compounds that facilitate entry of functional genes into cells, and whose activity in this regard is sufficient to provide for *in vivo* delivery of genes or other such biologically active  
25 therapeutic molecules in concentrations thereof that are sufficient for intracellular therapeutic effect.

### Reported Developments

In as much as compounds designed to facilitate intracellular delivery of biologically active molecules must interact with both non-polar and polar environments ( in or on, for example, the plasma membrane, tissue fluids, compartments within the cell, and the biologically active molecule itself ), such compounds are designed typically to contain both polar and non-polar domains. Compounds having both such domains may be termed amphiphiles, and many lipids and synthetic lipids that have been disclosed for use in facilitating such intracellular delivery (whether for *in vitro* or *in vivo* application) meet this definition. One particularly important class of such amphiphiles is the cationic amphiphiles. In general, cationic amphiphiles have polar groups that are capable of being positively charged at or around physiological pH, and this property is understood in the art to be important in defining how the amphiphiles interact with the many types of biologically active (therapeutic) molecules including, for example, negatively charged polynucleotides such as DNA.

Examples of cationic amphiphilic compounds that have both polar and non-polar domains and that are stated to be useful in relation to intracellular delivery of biologically active molecules are found, for example, in the following references, which contain also useful discussion of (1) the properties of such compounds that are understood in the art as making them suitable for such applications, and (2) the nature of structures, as understood in the art, that are formed by complexing of such amphiphiles with therapeutic molecules intended for intracellular delivery.

(1) Felgner, et al., Proc. Natl. Acad. Sci. USA, 84, 7413-7417 (1987) disclose use of positively-charged synthetic cationic lipids including N-[1(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride ("DOTMA"),

to form lipid/DNA complexes suitable for transfections. See also Felgner et al.,

The Journal of Biological Chemistry, 269(4), 2550-2561 (1994).

(2) Behr et al., Proc. Natl. Acad. Sci. USA, 86, 6982-6986 (1989) disclose  
5 numerous amphiphiles including dioctadecylamidoglycylspermine ("DOGS").

(3) U.S. Patent 5, 283,185 to Epand et al. describes additional classes and species of amphiphiles including 3ß [N-(N<sup>1</sup>,N<sup>1</sup> - dimethylaminoethane)-carbamoyl] cholesterol, termed "DC-chol".

10 (4) Additional compounds that facilitate transport of biologically active molecules into cells are disclosed in U.S. Patent No. 5,264,618 to Felgner et al. See also Felgner et al., The Journal Of Biological Chemistry, 269(4), pp. 2550- 2561 (1994) for disclosure therein of further compounds including  
15 "DMRIE" 1,2- dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide, which is discussed below.

(5) Reference to amphiphiles suitable for intracellular delivery of biologically active molecules is also found in U.S. Patent No. 5,334,761 to Gebeyehu et al., and in Felgner et al., Methods(Methods in Enzymology), 5, 67- 75 (1993).

20 Although the compounds mentioned in the above-identified references have been demonstrated to facilitate (although in many such cases only *in vitro* ) the entry of biologically active molecules into cells, it is believed that the uptake efficiencies provided thereby are insufficient to support numerous therapeutic applications, particularly gene therapy.

25 Additionally, since the above-identified compounds are understood to have only modest activity, substantial quantities thereof must be used leading to concerns about the toxicity of such compounds or of the metabolites thereof.

Accordingly there is a need to develop a "second generation" of cationic amphiphiles whose activity is so sufficient that successful therapies can be achieved therewith.



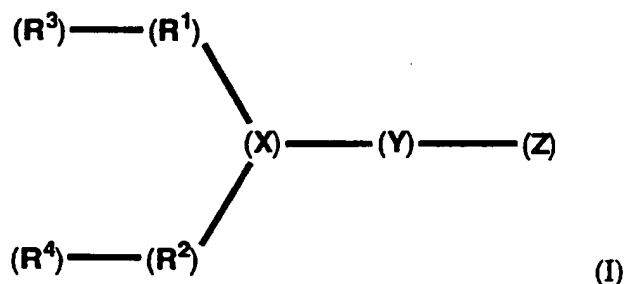
### Summary of the Invention

This invention provides for cationic amphiphiles that are particularly effective to facilitate transport of biologically active molecules into cells.

The cationic amphiphiles of the invention are divided into four (4) groups, although it will be seen that there are certain structural and functional features that many of the amphiphiles share.

Accordingly, there are provided cationic amphiphiles of Group I (see Figure 1, panels A, B, and C) capable of facilitating transport of biologically active molecules into cells, said amphiphiles having the structure (I),

10



wherein:

Z is a steroid;

15 X is a carbon atom or a nitrogen atom;

Y is a short linking group, or Y is absent;

R<sup>3</sup> is H, or a saturated or unsaturated aliphatic group;

R<sup>1</sup> is -NH-, an alkylamine, or a polyalkylamine;

R<sup>4</sup> is H, or a saturated or unsaturated aliphatic group;

20 R<sup>2</sup> is -NH-, an alkylamine, or a polyalkylamine;

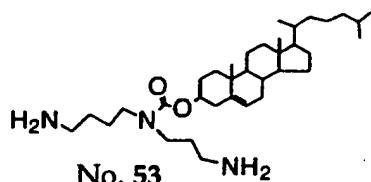
and wherein R<sup>1</sup> is the same or is different from R<sup>2</sup>, except that both R<sup>1</sup> and R<sup>2</sup> cannot be -NH-.

In one preferred embodiment, the steroid component "Z" is selected from the group consisting of 3-sterols, wherein said sterol molecule is linked by the 3-O- group thereof , or by N- in replacement thereof, to Y (or directly to X, if Y is absent ). According to this aspect of the invention, particularly effective amphiphiles include, for example, spermidine cholesterol carbamate ( N<sup>4</sup> -spermidine cholesteryl carbamate, amphiphile No. 53), and spermine cholesterol carbamate ( N<sup>4</sup>-spermine cholesteryl carbamate, amphiphile No. 67), and amphiphiles patterned thereon.

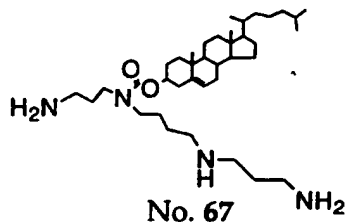
In a further preferred embodiment, the steroid group is linked to Y (or directly to X, if Y is absent) from ring position 17 of the steroid nucleus (see Figures 1 and 22), or from the arm that normally extends from position 17 in many steroids( see the structure of cholesterol in Figure 1), or from any shortened form of said arm.

In other preferred embodiments, within linking group Y are contained no more than about three or four atoms that themselves form a bridge of covalent bonds between X and Z. In a specific preferred embodiment of the invention, Y is a linking group wherein no more than one atom of said group forms a bond with both X and Z, or Y is absent.

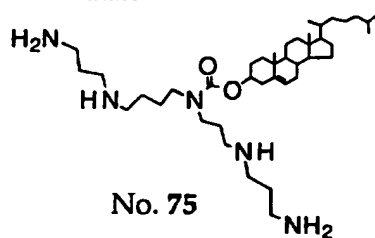
Representative amphiphiles provided according to Group I include:



$N^4$ -spermidine cholesteryl carbamate

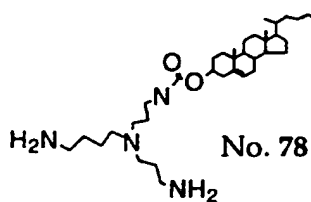


$N^4$ -spermine cholesteryl carbamate



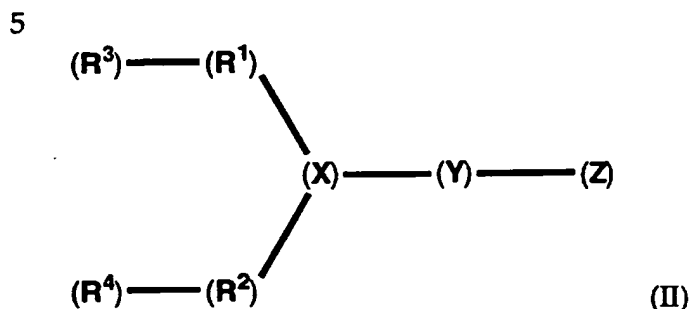
$N^1,N^8$ -Bis (3-aminopropyl)-  $N^4$ -spermidine cholesteryl carbamate

5



$N(N^4$ -3-aminopropylspermidine) cholesteryl carbamate

Additionally there are provided cationic amphiphiles of Group II (see Figure 5) capable of facilitating transport of biologically active molecules into cells said amphiphiles having the structure (II),



wherein:

Z is a steroid;

10 X is a carbon atom or a nitrogen atom;

Y is a linking group or Y is absent;

R³ is an amino acid, a derivatized amino acid, H or alkyl;

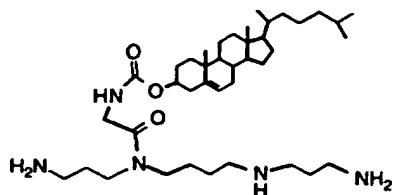
R¹ is -NH-, an alkylamine, or a polyalkylamine;

R⁴ is an amino acid, a derivatized amino acid, H or alkyl;

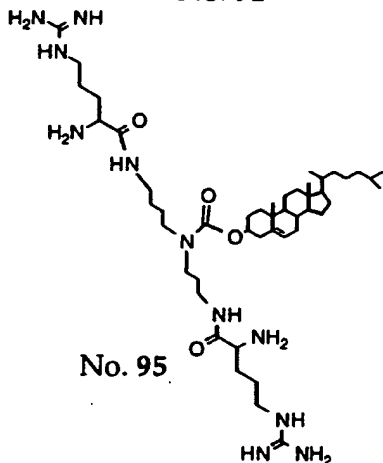
15 R² is -NH-, an alkylamine, or a polyalkylamine;

and wherein R¹ is the same or is different from R², except that both R¹ and R² cannot be -NH-.

Representative amphiphiles provided according to Group II include:



No. 91



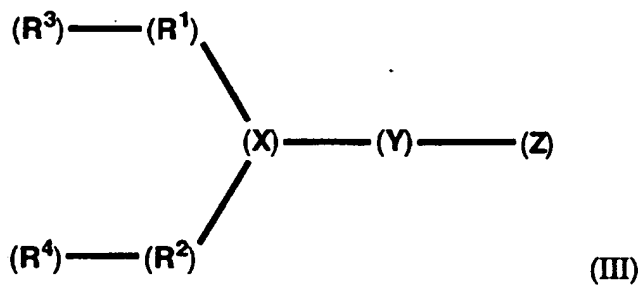
No. 95

$N^1, N^8$ -Bis(arginine carboxamide)-  
 $N^4$ -spermidine cholestery carbamate

5

Additionally there are provided cationic amphiphiles of Group III (see Figure 6) capable of facilitating transport of biologically active molecules into cells said amphiphiles having the structure (III),

10



wherein:

Z is an alkylamine or a dialkylamine, linked by the N-atom thereof, to Y ( or directly to X, if Y is absent ), wherein if Z is a dialkylamine, the alkyl groups thereof can be the same or different;

X is a carbon atom or a nitrogen atom;

5 Y is a short linking group , or Y is absent;

R<sup>3</sup> is H, or a saturated or unsaturated aliphatic group;

R<sup>1</sup> is -NH-- , an alkylamine, or a polyalkylamine;

R<sup>4</sup> is H, or a saturated or unsaturated aliphatic group;

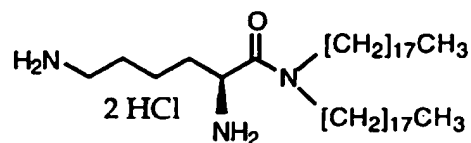
R<sup>2</sup> is -NH-- , an alkylamine, or a polyalkylamine;

10 and wherein R<sup>1</sup> is the same or is different from R<sup>2</sup> , except that both R<sup>1</sup> and R<sup>2</sup> cannot be -NH--.

With respect to amphiphiles provided according to Structure (III), it is again preferred that within linking group Y there are contained no more than about three or four atoms that themselves form a bridge of covalent

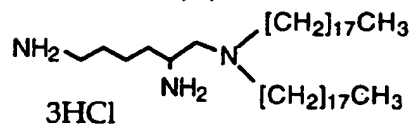
15 bonds between X and Z. In a specific preferred embodiment of the invention, Y is a linking group, such as > C=O, wherein no more than one atom of said group forms a bond with both X and Z, or Y is absent.

Representative amphiphiles provided according to Group III include: .



No. 43

N,N-dioctadecyllysineamide diHCl salt



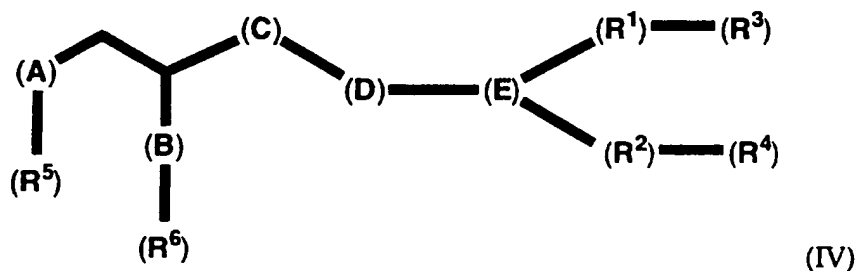
No. 47

N<sup>1</sup>,N<sup>1</sup>'-dioctadecyl-1,2,6-triaminohexane tri HCl salt

5

Additionally there are provided cationic amphiphiles of Group IV (see Figure 7) capable of facilitating transport of biologically active molecules into cells said amphiphiles having the structure (IV),

10



wherein:

A and B are independently O, N or S;

15 R<sup>5</sup> and R<sup>6</sup> are independently alkyl or acyl groups and may be saturated or contain sites of unsaturation;

C is selected from the group consisting of -CH<sub>2</sub>-, >C=O, and >C=S;

E is a carbon atom or a nitrogen atom;

D is a linking group such as  $\text{-NH(C=O)-}$  or  $\text{-O(C=O)-}$ , or D is absent;

$\text{R}^3$  is H, or a saturated or unsaturated aliphatic group;

$\text{R}^1$  is  $\text{-NH-}$ , an alkylamine, or a polyalkylamine;

5  $\text{R}^4$  is H, or a saturated or unsaturated aliphatic group;

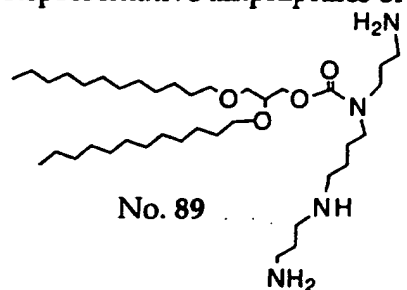
$\text{R}^2$  is  $\text{-NH-}$ , an alkylamine, or a polyalkylamine;

and wherein  $\text{R}^1$  is the same or is different from  $\text{R}^2$ , except that both  $\text{R}^1$  and

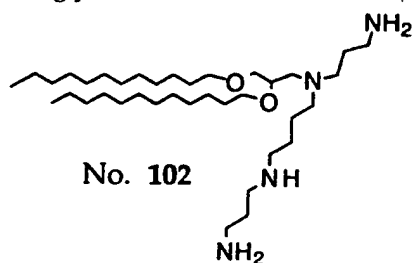
$\text{R}^2$  cannot be  $\text{-NH-}$ .



Representative amphiphiles of Group IV include:



1-(N<sup>4</sup>-spermine)-2,3-dilauryl-glycerol carbamate



N<sup>4</sup>-spermine-2,3-dilauryl-oxypropylamine

5           The invention provides also for pharmaceutical compositions that comprise one or more cationic amphiphiles, and one or more biologically active molecules, wherein said compositions facilitate intracellular delivery in the tissues of patients of therapeutically effective amounts of the biologically active molecules. The pharmaceutical compositions of the  
10 invention may be formulated to contain one or more additional physiologically acceptable substances that stabilize the compositions for storage and/or contribute to the successful intracellular delivery of the biologically active molecules.

15           In a further aspect, the invention provides a method for facilitating the transfer of biologically active molecules into cells comprising the steps of: preparing a dispersion of a cationic amphiphile of the invention; contacting said dispersion with a biologically active molecule to form a

complex between said amphiphile and said molecule, and contacting cells with said complex thereby facilitating transfer of said biologically-active molecule into the cells.

For pharmaceutical use, the cationic amphiphile(s) of the invention  
5 may be formulated with one or more additional cationic amphiphiles including those known in the art, or with neutral co-lipids such as dioleoylphosphatidyl-ethanolamine, ("DOPE"), to facilitate delivery to cells of the biologically active molecules. Additionally, compositions that comprise one or more cationic amphiphiles of the invention can be used to  
10 introduce biologically active molecules into plant cells, such as plant cells in tissue culture.

Additionally, the present application provides for novel plasmids suitable for complexing with the amphiphiles of the invention in order to treat patients by gene therapy, so that a high level of expression of the  
15 appropriate therapeutic transgene can be achieved. Representative examples thereof include the plasmid pCMVHI and pCFI. pCF1 plasmid contains the enhancer/promoter region from the immediate early gene of cytomegalovirus. The plasmid also contains a hybrid intron located between the promoter and the transgene cDNA. The polyadenylation signal of the  
20 bovine growth hormone gene was selected for placement downstream from the transgene. These and other features contribute substantially to the improved transgene expression possible with this plasmid.

Further enhancements in plasmid performance are made possible by the provision of replicating episomal plasmids. Additional therapeutic  
25 enhancements are made possible by providing plasmids in which expression of the therapeutic transgene is placed under the control of a transcriptional promoter that is sensitive to the concentration of

inflammation-related substances in the target tissue. Such plasmids are of particular use for the treatment of clinical cases in which inflammation is a major complication.

5 In a still further embodiment of the invention, particular organs or tissues may be targeted for gene therapy, by intravenous administration of amphiphile/transgene complexes, by adjusting the ratio of amphiphile to DNA in such complexes, and by adjusting the apparent charge or zeta potential thereof.

10 Further additional and representative aspects of the invention are described according to the Detailed Description of the Invention which follows directly.

### Brief Description of the Drawings

- FIGURE 1 depicts representative Group I cationic amphiphiles.
- FIGURE 2 depicts representative steroid lipophilic groups.
- FIGURE 3 depicts representative steroid lipophilic groups.
- 5    FIGURE 4 depicts a transacylation reaction.
- FIGURE 5 depicts representative Group II cationic amphiphiles.
- FIGURE 6 depicts representative Group III cationic amphiphiles.
- FIGURE 7 depicts representative Group IV cationic amphiphiles.
- FIGURE 8 provides a route of synthesis for spermidine cholesterol
- 10   carbamate.
- FIGURE 9 provides a route of synthesis for spermine cholesterol carbamate
- FIGURE 10 provides a comparison of in vivo transfection efficiency for certain cationic amphiphiles under particular conditions.
- FIGURE 11 is a depiction of in vivo transfection efficiency as a function of
- 15   DNA concentration for a particular cationic amphiphile.
- FIGURE 12 is a depiction of in vivo transfection efficiency as a function of concentration of a particular cationic amphiphile.
- FIGURE 13 provides relative transfection efficiencies for Group I amphiphiles.
- 20   FIGURE 14 provides relative transfection efficiencies for Group II amphiphiles.
- FIGURE 15 provides relative transfection efficiencies for Group IV amphiphiles.
- FIGURE 16 provides a map of pCMVHI-CAT plasmid.
- 25   FIGURE 17 shows the hybrid intron of pCMVHI-CAT.
- FIGURE 18 (panel A) provides a map of pCF1/CAT plasmid.
- FIGURE 18 (panel B) provides a map of pCF2/CAT plasmid.

FIGURE 19 shows a plot of corrected chloride ion transport in nasal polyp epithelial cells from a cystic fibrosis patient.

FIGURE 20 provides a map of pMyc4-CFTR plasmid.

FIGURE 21 demonstrates intravenous targeting of the heart and lung.

### Detailed Description of the Invention

#### Information Concerning the Structure of Cationic Amphiphiles of the Invention

This invention provides for cationic amphiphile compounds, and compositions containing them, that are useful to facilitate transport of biologically active molecules into cells. The amphiphiles are particularly useful in facilitating the transport of biologically active polynucleotides into cells, and in particular to the cells of patients for the purpose of gene therapy.

Cationic amphiphiles according to the practice of the invention possess several novel features. These features may be seen in comparison with, for example, cationic amphiphile structures such as those disclosed in U.S. Patent No. 5, 283,185 to Epand et al., a representative structure of which is 3 $\beta$  [N-(N<sup>1</sup>,N<sup>1</sup> - dimethylaminoethane)-carbamoyl] cholesterol, commonly known as "DC-chol", and to those disclosed by Behr et al. Proc. Natl. Acad. Sci. USA, 86, 6982- 6986 (1989), a representative structure of which is dioctadecylamidolo-glycylspermine ("DOGS").

Cationic amphiphiles of the present invention contain distinctive structural features: (1) the presence of a lipophilic group which is connected directly, or through a linking group, to two cationic groups (see below) that themselves comprise amino, alkylamine or polyalkylamine groups, there resulting an overall and novel "T-shaped" structure; and (2) in many cases, and in comparison with numerous art-recognized amphiphiles, the use of a relatively short linking group to bring into close proximity the lipophilic and cationic regions of the amphiphile. Without being limited as to theory, it is believed that these features contribute substantially to the transfection-enhancing capability of these compounds. As an example of this, Figure 10

below demonstrates the very substantial in vivo transfection-enhancing capability of spermidine cholesterol carbamate (a novel amphiphile of the invention) in comparison to DC- chol and DMRIE— two well recognized transfectants.

5           In connection with the practice of the present invention, it is noted that "cationic" means that the R groups, as defined herein, tend to have one or more positive charges in a solution that is at or near physiological pH. Such cationic character may enhance interaction of the amphiphile with  
10   plasma membrane glycoproteins) thereby contributing to successful entry of the therapeutic molecules into cells, or processing within subcompartments (such as the nucleus) thereof. In this regard, the reader is referred to the numerous theories in the art concerning transfection-enhancing function of cationic amphiphiles, none of which is to be taken as limiting on the practice  
15   of the present invention.

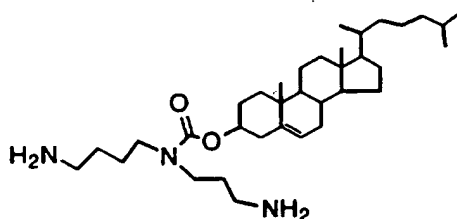
          Biological molecules for which transport into cells can be facilitated according to the practice of the invention include, for example, genomic DNA, cDNA, mRNA, antisense RNA or DNA, polypeptides and small  
20   molecular weight drugs or hormones. Representative examples thereof are mentioned below in connection with the description of therapeutic (pharmaceutical) compositions of the invention.

          In an important embodiment of the invention the biologically active molecule is an encoding polynucleotide that is expressed when placed in the cells of a patient leading to the correction of a metabolic defect. In a  
25   particularly important example, the polynucleotide encodes for a polypeptide having an amino acid sequence sufficiently duplicative of that of human cystic fibrosis transmembrane regulator ("CFTR") to allow

possession of the biological property of epithelial cell anion channel regulation.

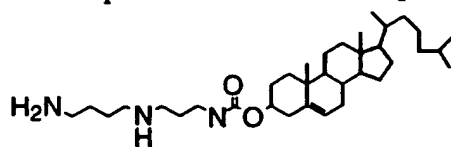
As aforementioned, characteristic and novel features of the amphiphiles of the invention include first, that the linking group that connects the two cationic amine groups to the lipophilic group is very short, or absent entirely, and second, that the resultant linking of the the two cationic R groups to the lipophilic group forms a T-shaped structure when viewed from the position of atom "X" (a carbon or nitrogen atom) as depicted, for example, in Structures (I), (II), (III) and (IV, see atom "E").

As examples of the cationic amphiphiles of the invention, both spermidine cholesterol carbamate ( N<sup>4</sup> -spermidine cholesteryl carbamate) and spermine cholesterol carbamate ( N<sup>4</sup> -spermine cholesteryl carbamate) have been determined to be superior transfectants *in vivo* in comparison with non "T-shaped" amphiphiles having otherwise equivalent amounts of cationic alkylamine structure. Superior performance (see also Example 3) has been determined for:



( spermidine cholesterol carbamate )

in comparison with, for example,

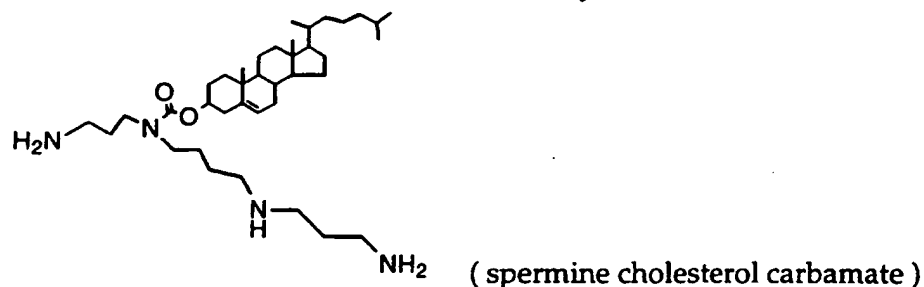


(N<sup>1</sup>-spermidine cholesteryl

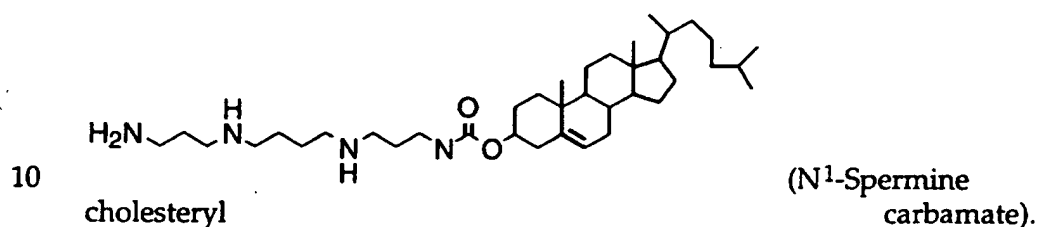
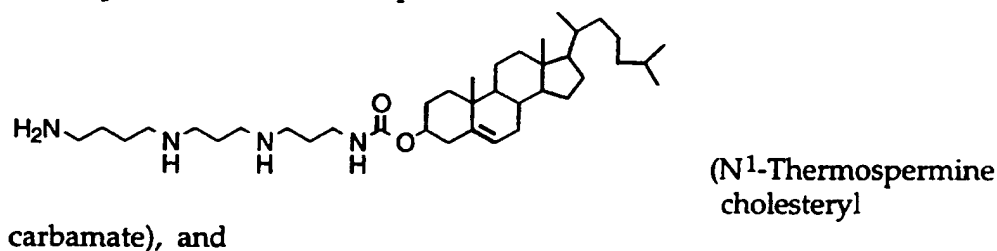
carbamate).



Additionally, superior performance has been determined for



5 in comparison with, for example,



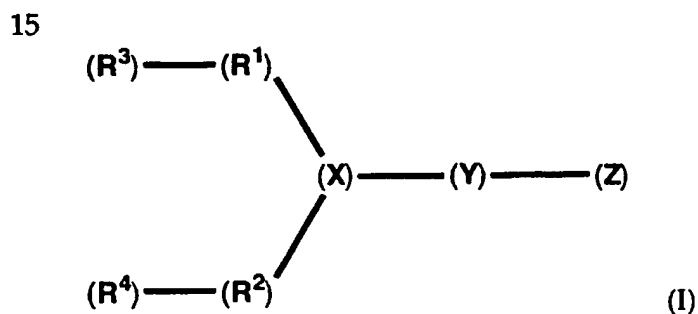
Applicants have also noted that numerous of the cationic  
15 amphiphiles of the invention have structural features in common with  
naturally occurring polyamines such as spermine and spermidine (including  
N-atom spacing). In this regard, the structures of amphiphiles 53, 67, 78, 90,  
and 91 are representative. As can be seen by examination of the data in  
Figures 13, 14 and 15, the placement of the nitrogen atoms in the polar head  
20 groups of the amphiphiles such that they are separated by one or more  
combinations of 3 and 4 carbon atoms leads to high in vivo transfection  
efficiency for plasmid transgenes complexed therewith. Applicants have

also noted that these in-common structural features may have a useful effect upon the binding of the amphiphiles to DNA, and on interaction with cell surface polyamine receptors. Interaction with cell polyamine receptors may be particularly important with respect to the treatment of cancer cells by gene therapy, since the DNA replication requirements of such cells may lead to high level expression of such receptors.

### Group I Amphiphiles

In connection with the design of the Group I amphiphiles of the invention, the following considerations are of note. Many of these design features are then discussed in connection with the other amphiphiles of the invention, those classified under Groups II, III and IV.

Accordingly, there are provided cationic amphiphiles of Group I (see Figure 1, panels A, B, and C) capable of facilitating transport of biologically active molecules into cells, said amphiphiles having the structure (I),



wherein:

Z is a steroid;

20 X is a carbon atom or a nitrogen atom;

Y is a short linking group, or Y is absent;

R<sup>3</sup> is H, or a saturated or unsaturated aliphatic group;

R<sup>1</sup> is -NH-, an alkylamine, or a polyalkylamine;

$R^4$  is H, or a saturated or unsaturated aliphatic group;

$R^2$  is  $-NH-$ , an alkylamine, or a polyalkylamine;

and wherein  $R^1$  is the same or is different from  $R^2$ , except that both  $R^1$  and  $R^2$  cannot be  $-NH-$ .

5 The Linking Group

Preferably the linking group that connects the lipophilic group to the two cationic R groups is relatively short. It is preferred that within linking group Y are contained no more than about three or four atoms that themselves form a bridge of covalent bonds between X and Z. Examples of

10 Y groups include --

$-(CH_2)_2-$ ;  $-(CH_2)_3-$ ;  $-(CH_2)-(C=O)-$ ;  $-(CH_2)_n-NH-(C=O)-$  where n is preferably 4 or less. Additional linking groups useful in the practice of the invention are those patterned on small amino acids such as glyciny, alanyl, beta-alanyl, seriny, and the like.

15 With respect to the above representations, the left hand side thereof as depicted- is intended to bond to atom "X", and the right hand side thereof to group "Z" ( see structure I).

In certain preferred embodiments of the invention, Y is a linking group wherein no more than one atom of this group forms a bond with both  
20 "X" and "Z". Examples of preferred linking groups include  $-CH_2-$ ,  $>C=S$ , and  $>C=O$ . Alternatively, the linking group "Y" may be absent entirely.

As aforementioned (see Structure I, directly above), "X" forms a connecting point in the amphiphiles to which is also attached the two cationic R groups. As can be seen therein (see also Figure 1), the placement  
25 of the nitrogen atom that represents "X" clearly causes the molecule to assume a T-shape.

### Steroid Lipophilic Groups

Cationic amphiphiles according to the practice of the invention may include a variety of structures as lipophilic group. Steroids represent a preferred group of such structures.

5           With respect to the design and orientation of steroids as lipophilic groups according to the practice of the invention, the following considerations are of note. Steroids are widely distributed in the animal, microbial and plant kingdoms. They may be defined as solid alcohols that typically contain, as their basic skeleton, 17 carbon atoms arranged in the  
10       form of a perhydrocyclopenteno-phenanthrene ring system. Accordingly, such compounds include bile acids, cholesterol and related substances, vitamin D, certain insect molting hormones, certain sex hormones, corticoid hormones, certain antibiotics, and derivatives of all of the above wherein additional rings are added or are deleted from the basic structure. [see  
15       Natural Products Chemistry, K. Nakanashi et al. eds., Academic Press, Inc., New York (1974), volume 1, at Chapter 6 for a further discussion of the broad classes of molecules that are understood in the art to be steroids]. Additionally, for the purposes of the invention, the term steroid is used broadly to include related molecules derived from multiple isoprenoid  
20       units, such as vitamin E. Steroids representative of those useful in the practice of the invention are shown in Figures 1, 2, 3 and 5.

As elaborated below, certain preferred amphiphiles of the invention include a steroid component "Z" that is selected from the group consisting of 3-sterols, wherein said sterol molecule is linked by the 3-O- group thereof  
25       , or by N- in replacement thereof, to Y (see Figure 1). Such structures include, for example, spermidine cholesterol carbamate, spermine cholesterol carbamate, spermidine 7-dehydrocholesteryl carbamate, lysine

3-N-dihydrocholesteryl carbamate, spermidine cholestamine urea, and N-3-aminopropyl-N-4-aminobutylcholestamine.

In a further preferred embodiment, the steroid group is linked to Y (or directly to X if Y is absent) from ring position 17 of the steroid nucleus (see Figures 1 and 3), or from the arm that normally extends from position 17 in many steroids (see Figures 1 and 3), or from any shortened form of said arm.

In connection with the selection of steroids for inclusion in the amphiphiles of the invention, it is preferred that the molecules have structures which can be metabolized by the body and are nontoxic at the doses thereof that are used. Preferred are steroids such as cholesterol and ergosterol that are substantially non toxic and which possess biologically normal stereospecificity in order to facilitate their safe metabolism in patients. Additional steroids useful in the practice of the invention include, for example, ergosterol B1, ergosterol B2, ergosterol B3, androsterone, cholic acid, desoxycholic acid, chenodesoxycholic acid, lithocholic acid and, for example, various derivatives thereof as are shown in the panels of Figures 2 and 3.

With respect to the orientation of the steroid lipophilic group, that is, how the group is attached(with or without a linker) to the cationic (alkyl) amine groups of an amphiphile, the following further information is of note. Any ring position or substituent on the steroid can in general be used as point of attachment. It is preferred, however, to use a point of attachment that (1) minimizes the complexity of chemical syntheses, and (2) is positioned near either "end" of the steroid molecule, for example , a position near ring position 3, or near ring position 17( or the arm that typically extends therefrom). Such positions provide an orientation of the

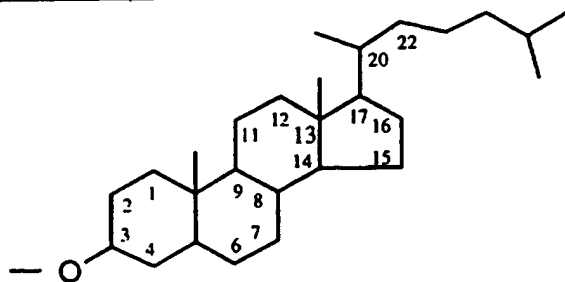
steroid with respect to the rest of the amphiphile structure that facilitates bilayer formation, and/or micelle formation, and/or stabilizes interaction with the biologically active molecules to be carried into the target cells.

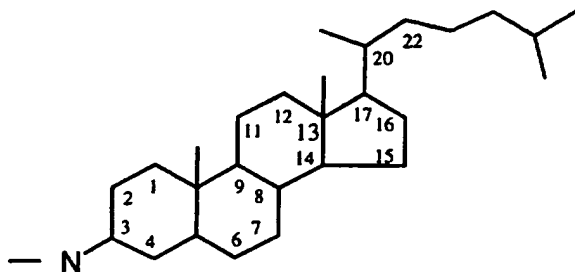
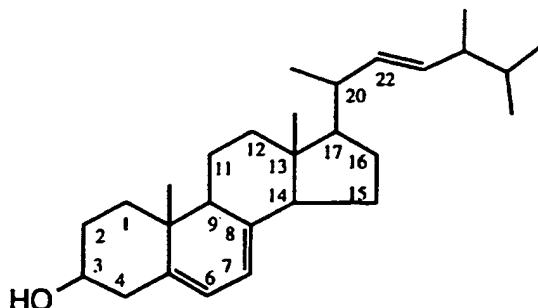
Representative structures showing attachment of the cationic (alkyl) amine groups to the steroid lipophilic group through the arm extending from ring position 17 thereof are shown in Figure 3 (panels A, B). With respect to this type of structure, it is further preferred that any polar groups on the steroid, such as may be attached to ring position 3, be either removed or capped (for example, hydroxy as methoxy) to avoid potentially destabilizing bilayer or micelle structures.

The representation in Figure 3 of cationic amphiphiles in which the steroid lipophilic group thereof is linked to the cationic alkylamine groups through steroid ring position 17 is but an example of the invention. Similarly, the representation in Figures 1 to 4 of cationic amphiphiles in which the steroid lipophilic group thereof is linked to the cationic alkylamine groups through steroid ring position 3 is an example of the invention. As aforementioned, use of any steroid ring position (or moiety or branch extending therefrom) as point of attachment is within the practice of the invention.

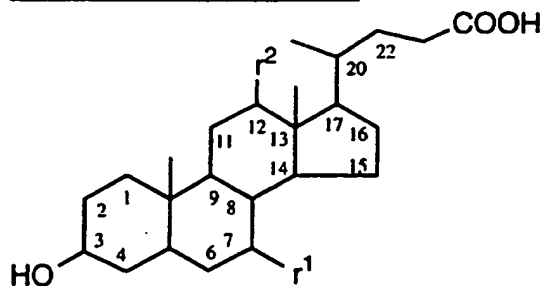
Preferred steroids for use as group "Z" according to the practice of the invention include:

3- sterols (derived from cholesterol)



3-N steryl groups (patterned on cholesterol)5 ergosterol and derivatives

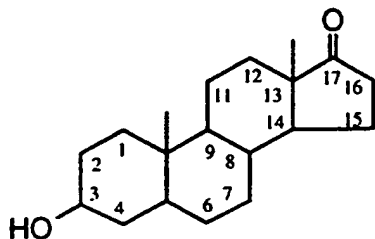
Representative species of steroid that are patterned on ergosterol and that may be used to define the structure of cationic amphiphiles of the invention include: ergosterol (double bonds as shown); ergosterol B1 ( $\Delta$  8, 9;  $\Delta$  14, 15;  $\Delta$  22, 23); ergosterol B1 ( $\Delta$  6, 7;  $\Delta$  8, 14;  $\Delta$  22, 23); ergosterol B1 ( $\Delta$  7, 8;  $\Delta$  14, 15;  $\Delta$  22, 23); and lumisterol ( the 9b-H isomer of ergosterol).

cholic acid and derivatives

15

Representative species of steroid that are patterned on cholic acid and that may be used to define the structure of cationic amphiphiles of the invention include: cholic acid wherein  $r^1$  and  $r^2 = \text{OH}$ ; desoxycholic acid wherein  $r^1 = \text{H}$  and  $r^2 = \text{OH}$ ; chenodesoxycholic acid wherein  $r^1 = \text{OH}$  and  $r^2 = \text{H}$ ; and lithocholic acid wherein  $r^1$  and  $r^2 = \text{H}$ .

androsterone and derivatives thereof



Selection of Groups  $R^1$ ,  $R^2$ ,  $R^3$ , and  $R^4$

For  $R^3$  and  $R^4$ :

According to the practice of the invention  $R^3$  and  $R^4$  are, independently, H, or saturated or unsaturated aliphatic groups. The aliphatic groups can be branched or unbranched. Representative groups include alkyl, alkenyl, and cycloalkyl.

For  $R^1$  and  $R^2$ :

$R^1$  and  $R^2$  represent structures recognized in the art as being amine; alkylamines (including primary, secondary, and tertiary amines), or extended versions thereof—herein termed “polyalkylamines”. It is understood that both alkylamine and polyalkylamine groups as defined herein may include one or more carbon-carbon double bonds and the use of such alkenylamines is therefore within the practice of the invention.



Representative alkylamines include: (a)  $-\text{NH}-(\text{CH}_2)_z-$  where  $z$  is other than 0; (b)  $-[\text{CH}_3(\text{CH}_2)_y\text{N}](\text{CH}_2)_z-$  where  $z$  is other than 0; and (c)  $-[\text{CH}_3(\text{CH}_2)_x][\text{CH}_3(\text{CH}_2)_y]\text{N}-(\text{CH}_2)_z-$  where  $z$  is other than 0.

With respect to the circumstance where one or both of  $\text{R}^1$  and  $\text{R}^2$  are tertiary amines, such as is represented in Structure (c) above, it is understood that a hydrogen atom corresponding to either  $\text{R}^3$  or  $\text{R}^4$ , as appropriate, may or may not be present since such hydrogen atoms correspond to the  $\text{N:H}(+)$  structure whose level of protonation will vary according to pH.

The term "polyalkylamine" as referred to herein defines a polymeric structure in which at least two alkylamines are joined. The alkylamine units that are so joined may be primary or secondary, and the polyalkylamines that result may contain primary, secondary, or tertiary N-atoms. The alkylamine (sub)units may be saturated or unsaturated, and therefore the term "alkylamine" encompasses alkenylamines in the description of the invention.

Representative resultant polyalkylamines include: (d)  $-\text{NH}-(\text{CH}_2)_z]_q-$ , where  $z$  is other than 0, and  $q$  is 2 or higher; (e)  $-\text{NH}-(\text{CH}_2)_y]_p-\text{NH}-(\text{CH}_2)_z]_q-$ , where  $y$  and  $z$  are each other than 0, and  $p$  and  $q$  are each other than 0; (f)  $-\text{NH}-(\text{CH}_2)_x]_n-\text{NH}-(\text{CH}_2)_y]_p-\text{NH}-(\text{CH}_2)_z]_q-$ , where  $x$ ,  $y$ , and  $z$  are each other than 0, and  $n$ ,  $p$  and  $q$  are each other than 0; (g)  $-\text{NH}-(\text{CH}_2)_w]_m-\text{NH}-(\text{CH}_2)_x]_n-\text{NH}-(\text{CH}_2)_y]_p-\text{NH}-(\text{CH}_2)_z]_q-$ , where  $w$ ,  $x$ ,  $y$ , and  $z$  are each other than 0, and  $m$ ,  $n$ ,  $p$ , and  $q$  are each other than 0; (h)  $-\text{NH}-(\text{CH}_2)_w]_m-\text{NH}-(\text{CH}_2)_x]_n-[\text{CH}_3(\text{CH}_2)_y\text{N}](\text{CH}_2)_z-$ , where  $x$ ,  $n$  and  $z$  are each other than 0; (i)  $-\text{NH}-(\text{CH}_2)_w]_p-[\text{CH}_3(\text{CH}_2)_x\text{N}](\text{CH}_2)_y-\text{NH}-(\text{CH}_2)_z]_q-$ , where  $w$ ,  $p$ ,  $y$ ,  $z$ , and  $q$  are each other than 0; and (j)

$-\text{[NH-(CH}_2\text{)}_v\text{]}_l - \text{[NH-(CH}_2\text{)}_w\text{]}_m - \text{[NH-(CH}_2\text{)}_x\text{]}_n - \text{[NH-(CH}_2\text{)}_y\text{]}_p$   
 $-\text{[NH-(CH}_2\text{)}_z\text{]}_q -$ , where  $v, w, x, y$ , and  $z$  are each other than 0, and  $l, m, n, p$ , and  $q$  are each other than 0.

As mentioned above  $R^1$  and  $R^2$ , independently, can be  $-\text{NH}-$ , an  
 5 alkylamine, or a polyalkylamine, and can be the same or different from each  
 other, except that both  $R^1$  and  $R^2$  cannot be  $-\text{NH}-$  in order to (1) preserve  
 the "T- shape" of the resultant compound, and (2) to provide for the  
 stability thereof. It is preferred that - in combination- the combined  
 backbone length of  $R^3R^1$  (or of  $R^4R^2$ ) be less than about 40 atoms of  
 10 nitrogen and carbon, more  
 preferably less than about 30 atoms of nitrogen and carbon.

In the case where the  $R^1$  group adjacent to  $R^3$  (or  $R^2$  adjacent to  $R^4$ )  
 includes a terminal nitrogen atom that defines a tertiary center, then a  
 quaternary amine is formed (at that nitrogen atom of  $R^1$ ) if  $R^3$  is an  
 15 aliphatic group, and a tertiary amine remains (at that nitrogen atom of  $R^1$ ) if  
 $R^3$  is H. Accordingly, with respect to such resultant  $R^3R^1$  or  $R^4R^2$   
 structures, representative respective formulas are:  
 (k)  $\text{H-(CH}_2\text{)}_w - \text{[CH}_3\text{(CH}_2\text{)}_x\text{][CH}_3\text{(CH}_2\text{)}_y\text{]N} - \text{(CH}_2\text{)}_z -$ , where  $w$  and  
 $z$  are each other than zero; and (l)  $\text{H} - \text{[CH}_3\text{(CH}_2\text{)}_x\text{][CH}_3\text{(CH}_2\text{)}_y\text{]N} -$   
 20  $\text{(CH}_2\text{)}_z -$ , where  $z$  is other than zero.

In connection with interpreting the structural diagrams described  
 herein, it is intended that the attachment of  $R^3R^1-$  (or  $R^4R^2-$ ) structures  
 to atom "X" is through the right hand side (as depicted) of the  $R^3R^1-$ , that  
 is, through a  $\text{CH}_2-$  moiety. The coefficients (i.e.  $v, w, x, y$ , and  $z$  and  $l, m, n,$   
 25  $p$ , and  $q$ ) as depicted herein represent whole numbers. For the purposes of  
 the invention, "whole number" means 0 and the natural numbers  
 1,2,3,4,5,6.....and up, unless specifically restricted.

With respect to the amphiphiles of the invention including those represented by formulas (a) to (l), it is noted that there are certain preferences concerning the design of such groups depending on whether atom 'X' as it is shown according to structure (I) above, is a nitrogen atom

5 or a carbon atom. If "X" is nitrogen, then amphiphiles containing  $R^3-R^1$  ( or  $R^4-R^2$  ) groups that end in an N atom [ i.e formula (e) where z equals 0 and q=1; formula (h) where z equals 0] are not preferred, since the resultant N-N linkage involving position X results in an amphiphile that may be unstable and/or difficult to prepare. An additional group of structures that

10 are difficult to prepare and/or are unstable is represented, for example, by the R sequence (whether in  $R^1$ , or bridging  $R^1$  and  $R^3$ )  $--NH-CH_2-NH-CH_2--$ . Accordingly, use of such structures [ i.e. formula (a) where Z equals 1, formula (e) where one or both of y and z equals 1] in the practice of the invention is not preferred.

15 With respect to the design of structures (such as those depicted above) for inclusion in cationic amphiphiles, the following further considerations are of note. Any combination of alternating amine and alkyl moieties creates an R structure within the scope of the invention. A polyalkylamine may be represented, for example, by the formulas above,

20 although many more structures (such structures being within the scope of the invention) can be depicted by extending the number of, or types or combinations of, alkylamine subunits within the amphiphile structure. That further such variations can be made is apparent to those skilled in the art.

25 It is noted that a polyalkylamine group (or resultant  $R^3R^1$  group) that is very long may interfere, for example, with the solubility of the resultant amphiphile, or interfere with its ability to stably interact with the

biologically active molecule selected for intracellular delivery. In this regard, polyalkylamines (or resultant  $R^3R^1$  groups) having a backbone length of about 40 nitrogen and carbon atoms, or more, may not be suitable for inclusion in amphiphiles. However, for each such proposed structure,

5 its properties may be determined by experimentation, and its use is nonetheless within the practice of the invention.

Accordingly, specific alkylamine and polyalkylamine structures result as follows:

Table 1

<u>For R<sup>1</sup> and/or</u>	
5	<u>R<sup>2</sup></u>
	(1) -NH-
	(2) -NH-(CH <sub>2</sub> ) <sub>(2)</sub> -
	(3) -NH-(CH <sub>2</sub> ) <sub>(3)</sub> -
	(4) -NH-(CH <sub>2</sub> ) <sub>(4)</sub> -
10	(5) -NH-(CH <sub>2</sub> ) <sub>(6)</sub> -
	(6) -NH-(CH <sub>2</sub> ) <sub>(3)</sub> -NH-(CH <sub>2</sub> ) <sub>(4)</sub> -
	(7) -NH-(CH <sub>2</sub> ) <sub>(2)</sub> -NH-(CH <sub>2</sub> ) <sub>(2)</sub> -
	(8) -NH-(CH <sub>2</sub> ) <sub>(4)</sub> -NH-(CH <sub>2</sub> ) <sub>(3)</sub> -
	(9) -NH-(CH <sub>2</sub> ) <sub>(y)</sub> -NH-(CH <sub>2</sub> ) <sub>(z)</sub> -
15	(10) -NH-(CH <sub>2</sub> ) <sub>(x)</sub> -NH-(CH <sub>2</sub> ) <sub>(y)</sub> -NH-(CH <sub>2</sub> ) <sub>(z)</sub> -
	(11) -NH-(CH <sub>2</sub> ) <sub>(w)</sub> -NH-(CH <sub>2</sub> ) <sub>(x)</sub> -NH-(CH <sub>2</sub> ) <sub>(y)</sub> -NH-(CH <sub>2</sub> ) <sub>(z)</sub> -
	(12) -NH-(CH <sub>2</sub> ) <sub>(v)</sub> -NH-(CH <sub>2</sub> ) <sub>(w)</sub> -NH-(CH <sub>2</sub> ) <sub>(x)</sub> -NH-(CH <sub>2</sub> ) <sub>(y)</sub> -NH-(CH <sub>2</sub> ) <sub>(z)</sub> -
	(13) -[NH-(CH <sub>2</sub> ) <sub>(w)</sub> ] <sub>m</sub> - [NH-(CH <sub>2</sub> ) <sub>(x)</sub> ] <sub>n</sub> - [[CH <sub>3</sub> (CH <sub>2</sub> ) <sub>y</sub> ]N] - (CH <sub>2</sub> ) <sub>z</sub> -
20	(14) -[NH-(CH <sub>2</sub> ) <sub>(x)</sub> ] <sub>n</sub> - [[CH <sub>3</sub> (CH <sub>2</sub> ) <sub>y</sub> ]N] - (CH <sub>2</sub> ) <sub>z</sub> -
	(15) -[NH-(CH <sub>2</sub> ) <sub>(w)</sub> ] <sub>m</sub> - [NH-(CH <sub>2</sub> ) <sub>(x)</sub> ] <sub>n</sub> - [[CH <sub>3</sub> (CH <sub>2</sub> ) <sub>y</sub> ]N] - (CH <sub>2</sub> ) <sub>z</sub> -
	(16) - [[CH <sub>3</sub> (CH <sub>2</sub> ) <sub>x</sub> ][CH <sub>3</sub> (CH <sub>2</sub> ) <sub>y</sub> ]N] - (CH <sub>2</sub> ) <sub>z</sub> -
	(17) -NH-(CH <sub>2</sub> ) <sub>(z)</sub> -NH-
	(18) -NH-(CH <sub>2</sub> ) <sub>(y)</sub> -NH-(CH <sub>2</sub> ) <sub>(z)</sub> -NH-
25	(19) -NH-(CH <sub>2</sub> ) <sub>(y)</sub> -CH=CH-(CH <sub>2</sub> ) <sub>z</sub> -
	(20) - [NH-(CH <sub>2</sub> ) <sub>(w)</sub> ] <sub>p</sub> - [[CH <sub>3</sub> (CH <sub>2</sub> ) <sub>x</sub> ]N] - (CH <sub>2</sub> ) <sub>y</sub> - [NH-(CH <sub>2</sub> ) <sub>(z)</sub> ] <sub>q</sub> -

For  $R^3$  and/or

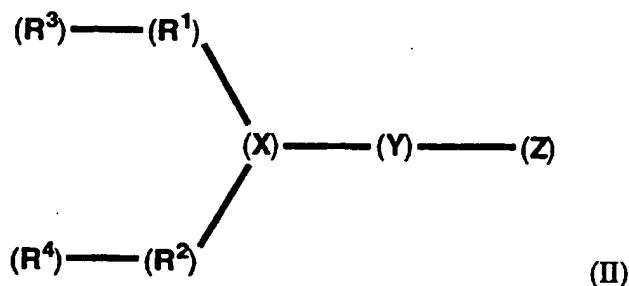
$R^4$  \_\_\_\_\_

- (1) H-
- (2) CH<sub>3</sub>-
- 5 (3) CH<sub>3</sub>-(CH<sub>2</sub>)<sub>2</sub>-
- (4) CH<sub>3</sub>-(CH<sub>2</sub>)<sub>4</sub>-
- (5) CH<sub>3</sub>-(CH<sub>2</sub>)<sub>z</sub>-
- (6) CH<sub>3</sub>-[CH<sub>3</sub>-(CH<sub>2</sub>)<sub>z</sub>]CH -
- (7) CH<sub>3</sub>-[CH<sub>3</sub>-(CH<sub>2</sub>)<sub>2</sub>]CH -
- 10 (8) CH<sub>3</sub>-[[CH<sub>3</sub>-(CH<sub>2</sub>)<sub>y</sub>][CH<sub>3</sub>-(CH<sub>2</sub>)<sub>z</sub>]]C -
- (9) CH<sub>3</sub>-(CH<sub>2</sub>)<sub>z</sub>-CH=CH-CH<sub>2</sub> -
- (10) CH<sub>3</sub>-[CH<sub>3</sub>-(CH<sub>2</sub>)<sub>y</sub>-CH=CH-(CH<sub>2</sub>)<sub>z</sub>]CH -
- (11) CH<sub>3</sub>-[[CH<sub>3</sub>-(CH<sub>2</sub>)<sub>w</sub>-CH=CH-(CH<sub>2</sub>)<sub>x</sub>][CH<sub>3</sub>-(CH<sub>2</sub>)<sub>y</sub>-CH=CH-(CH<sub>2</sub>)<sub>z</sub>]]CH -
- 15 (12) CH<sub>3</sub>-[CH<sub>3</sub>-(CH<sub>2</sub>)<sub>y</sub>]CH-(CH<sub>2</sub>)<sub>z</sub> -

**Group II Amphiphiles**

Additionally there are provided cationic amphiphiles of Group II (see Figure 5) capable of facilitating transport of biologically active molecules into cells said amphiphiles having the structure (II),

5



wherein:

Z is a steroid;

10 X is a carbon atom or a nitrogen atom;

Y is a linking group or Y is absent;

R<sup>3</sup> is an amino acid, a derivatized amino acid, H or alkyl;

R<sup>1</sup> is -NH-, an alkylamine, or a polyalkylamine;

R<sup>4</sup> is an amino acid, a derivatized amino acid, H or alkyl;

15 R<sup>2</sup> is -NH-, an alkylamine, or a polyalkylamine;

and wherein R<sup>1</sup> is the same or is different from R<sup>2</sup>, except that both R<sup>1</sup> and R<sup>2</sup> cannot be -NH-.

Representative amphiphiles provided according to Group II include amphiphiles 87, 91, 93, 95, 97, 99, 100, and 103. With respect to the structural

20 features of these amphiphiles, and the other amphiphiles of Group II, the following should be considered.

The steroid group may be selected according to the criteria defined above for the Group I amphiphiles. Accordingly, preferred amphiphiles include those selected from 3- sterols, wherein the sterol molecule is linked by the 3-O- group thereof, or by N in replacement thereof, to "Y".

- 5           The linking group Y of the Group II amphiphiles consists of an N-acylamino acid (or a derivative thereof), or consists of a group (such as > C=O or > C=S) wherein no more than one atom of said group forms a bond with both "X" and "Z". Optionally, group Y may be absent. Representative N-acylamino groups include an N-Acyl serine ( No. 87), an N-Acyl glycine  
10 (No. 91), and an N-Acyl aspartic acid ( No. 103). With respect to the use of N-Acyl aspartic acid in amphiphile No. 103, it is noted that, as provided, the gamma carboxyl thereof is further derivatized to an additional alkylamine moiety.

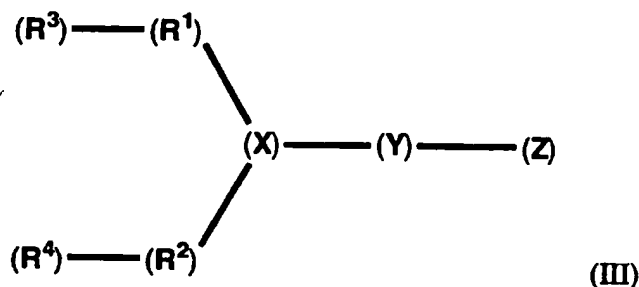
- The criteria for selection of  $R^1$  and  $R^2$  are as set forth for the Group I  
15 amphiphiles.  $R^3$  and  $R^4$  represent H or alkyl, or may be natural or artificial amino acids including derivatives of either. Representative examples of  $R^3$  or  $R^4$  amino acid groups include those derived from tryptophan ( No. 97) and from arginine ( No. 95).



**Group III Amphiphiles**

Additionally there are provided cationic amphiphiles of Group III (see Figure 6) capable of facilitating transport of biologically active molecules into cells said amphiphiles having the structure (III),

5



wherein:

- Z is an alkylamine or a dialkylamine, linked by the N-atom thereof, to Y, or directly to X if Y is absent, wherein if Z is a dialkylamine, the alkyl groups thereof can be the same or different;
- X is a carbon atom or a nitrogen atom;
- Y is a short linking group, or Y is absent;
- R<sup>3</sup> is H, or a saturated or unsaturated aliphatic group;
- R<sup>1</sup> is --NH-- , an alkylamine, or a polyalkylamine;
- 15 R<sup>4</sup> is H, or a saturated or unsaturated aliphatic group;
- R<sup>2</sup> is --NH-- , an alkylamine, or a polyalkylamine;
- and wherein R<sup>1</sup> is the same or is different from R<sup>2</sup> , except that both R<sup>1</sup> and R<sup>2</sup> cannot be --NH--.

Representative cationic amphiphiles according to the practice of the invention that contain an alkyl amine or dialkylamine as lipophilic group include, for example, N,N-dioctadecyllysineamide; N<sup>1</sup>, N<sup>1</sup>-dioctadecyl-1,2,6- triaminohexane; N,N-didodecyllysineamide; N,N-didodecyllysineamide; spermidine- N,N- dioctadecyl urea; N-

myristyllysineamide; and N-(dioctyldecylaminoethyl)-lysineamide .

Representative amphiphiles are depicted (Figure 6) as amphiphiles 43, 47, 56, 60, and 73. With respect to the structural features of these amphiphiles, and the other amphiphiles of Group III, the following should be considered.

- 5           With respect to the selection of the lipophilic alkylamine or dialkylamine group "Z", Table 2 below provides representative structures.

Table 2

For "Z"

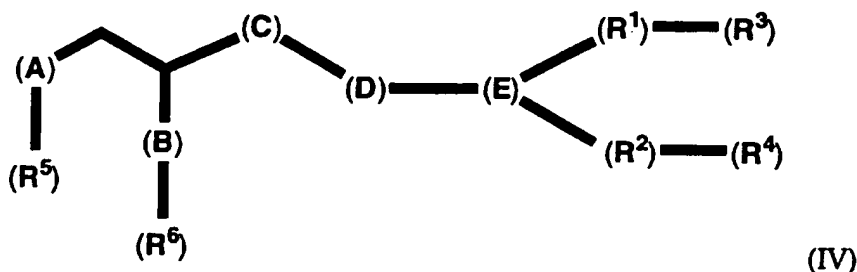
- 
- 10   (1)  $\text{CH}_3\text{-(CH}_2\text{)}_{13}\text{-NH-}$   
       (2)  $\text{CH}_3\text{-(CH}_2\text{)}_z\text{-NH-}$   
       (3)  $[[\text{CH}_3(\text{CH}_2)_{17}][\text{CH}_3(\text{CH}_2)_{17}]]\text{N-}$   
       (4)  $[[\text{CH}_3(\text{CH}_2)_{11}][\text{CH}_3(\text{CH}_2)_{11}]]\text{N-}$   
       (5)  $[[\text{CH}_3(\text{CH}_2)_9][\text{CH}_3(\text{CH}_2)_9]]\text{N-}$   
   15   (6)  $[[\text{CH}_3(\text{CH}_2)_x][\text{CH}_3(\text{CH}_2)_y]]\text{N-}$   
       (7)  $[[\text{CH}_3(\text{CH}_2)_x][\text{CH}_3(\text{CH}_2)_y\text{CH=CH(CH}_2\text{)}_z]]\text{N-}$   
       (8)  $[[\text{CH}_3(\text{CH}_2)_w][\text{CH}_3(\text{CH}_2)_x\text{CH=CH(CH}_2\text{)}_y\text{CH=CH(CH}_2\text{)}_z]]\text{N-}$

- In connection with the selection of suitable alkylamine or dialkylamine groups for inclusion at position Z in the amphiphiles of the invention, an alkyl chain(s) of the group should not be so large in molecular weight that it interferes with the solubility of the amphiphile, or interferes with its ability to interact with plasmid DNA. Additionally, an alkyl chain of an alkylamine or dialkylamine may include one or more points of unsaturation. The selection of R groups  $\text{R}^1$ ,  $\text{R}^2$ ,  $\text{R}^3$ , and  $\text{R}^4$  follows that disclosed for the Group I amphiphiles, and these R groups may be selected, for example, from Table I. Linking group Y may be selected as for the Group I amphiphiles, and preferred examples thereof include  $\text{—CH}_2\text{—}$ , and  $>\text{C=O}$ .
- 25

**Group IV Amphiphiles**

Additionally there are provided cationic amphiphiles of Group IV (see Figure 7) capable of facilitating transport of biologically active molecules into cells said amphiphiles having the structure (IV),

5



wherein:

A and B are independently O, N or S;

- 10 R<sup>5</sup> and R<sup>6</sup> are independently alkyl or acyl groups and may be saturated or contain sites of unsaturation;

C is selected from the group consisting of -CH<sub>2</sub>-, >C=O, and >C=S;

E (analogous to "X" in structures I, II, III) is a carbon atom or a nitrogen atom;

- 15 D is a linking group such as -NH(C=O)- or -O(C=O)-, or D is absent;

R<sup>3</sup> is H, or a saturated or unsaturated aliphatic group;

R<sup>1</sup> is -NH-, an alkylamine, or a polyalkylamine;

R<sup>4</sup> is H, or a saturated or unsaturated aliphatic group;

R<sup>2</sup> is -NH-, an alkylamine, or a polyalkylamine;

- 20 and wherein R<sup>1</sup> is the same or is different from R<sup>2</sup>, except that both R<sup>1</sup> and R<sup>2</sup> cannot be -NH-.

Representative amphiphiles of Group IV include Nos. 64, 76, 85, 89, 94, 98, 102, 105, 110, and 111. With respect to the structural features of these

amphiphiles, and the other amphiphiles of Group IV, the following should be considered.

With respect to the selection of  $R^1$ ,  $R^2$ ,  $R^3$ , and  $R^4$ , the teachings provided for Group I, II, and III amphiphiles are applicable. As  
5   aforementioned, group "E" represents a carbon atom or a nitrogen atom.

$R^5$  and  $R^6$  are independently alkyl or acyl groups, preferably containing about 8 to about 30 carbon atoms, and such groups may contain one or more points of unsaturation.

With respect to the selection of Group D, linkers such as  $-NH(C=O)-$   
10   or  $-O(C=O)-$  are preferred, and are depicted such that the left side thereof is intended to bond to "C" and the right side thereof is intended to bond to "E". Optionally, group D may be absent (amphiphile No.94). Additional linkers may be selected based on the teachings provided with respect to Groups I, II, and III above, and based upon the in vivo test data derived (  
15   Figure 15), it is preferred that the linker D be short or absent.

#### Co-lipids

Representative co-lipids that are useful according to the practice of the invention for mixing with one or more cationic amphiphiles include dioleoylphosphatidylethanolamine ("DOPE"), diphytanoylphosphatidyl-  
20   ethanolamine, lyso-phosphatidylethanolamines other phosphatidyl-ethanolamines, phosphatidylcholines, lyso-phosphatidylcholines and cholesterol. Typically, a preferred molar ratio of cationic amphiphile to colipid is about 1:1. However, it is within the practice of the invention to vary this ratio (see Example 3 below), including also over a considerable  
25   range.

It is generally believed in the art that preparing cationic amphiphiles as complexes with co-lipids (particularly neutral co-lipids) enhances the

capability of the amphiphile to facilitate transfections. Although colipid-enhanced performance has been observed for numerous of the amphiphiles of the invention, the amphiphiles of the invention are active as transfectants without co-lipid. Accordingly, the practice of the present invention is  
5 neither to be considered limited by theories as to co-lipid participation in intracellular delivery mechanisms, nor to require the involvement of co-lipids.

#### Transacylation Reactions

Although heretofore unrecognized in the art, it has been determined  
10 also that certain co-lipids may react chemically with certain types of cationic amphiphiles under conditions of co-storage, there resulting new molecular species. Generation of such new species is believed to occur via mechanisms such as transacylation. In this regard, see Figure 4 which depicts a transacylation reaction involving spermine cholesterol  
15 carbamate(No.67) and DOPE, there resulting lyso PE species and multiple forms of particular acyl- cationic amphiphile ( designated No. 80).

With respect to such reactions, the following remarks are of interest. With respect to use of amphiphile No.67, it has been observed that a mixture of amphiphile and DOPE, in chloroform solvent, does not appear to  
20 participate in such reactions. However, preparing the amphiphile and co-lipid in an aqueous solution where bilayer-containing structures such as liposomes can form will permit transacylation. Additionally, if amphiphile and co-lipid are dried down to a thin film, such as from chloroform (thereby placing the 2 species in intimate contact), then transacylation also occurs,  
25 possibly as a result of entropic effects. It is expected that these phenomena would also apply to lyophilized amphiphile/DOPE preparations.

Accordingly, it is highly preferred to maintain such amphiphile /DOPE preparations at very cold temperatures, such as -70 degrees C. Preparation of amphiphile No. 67 as a mono, di, or tri acetate salt has also been determined to slow transacylations.

- 5           It is to be understood that therapeutically-effective pharmaceutical compositions of the present invention may or may not contain such transacylation byproducts, or other byproducts, and that the presence of such byproducts does not prevent the therapeutic use of the compositions containing them. Rather use of such compositions is within the practice of
- 10   the invention, and such compositions and the novel molecular species thereof are therefore specifically claimed.

Preparation of Pharmaceutical Compositions and Administration Thereof

- The present invention provides for pharmaceutical compositions that facilitate intracellular delivery of therapeutically effective amounts of
- 15   biologically active molecules. Pharmaceutical compositions of the invention facilitate entry of biologically active molecules into tissues and organs such as the gastric mucosa, heart, lung, and solid tumors. Additionally, compositions of the invention facilitate entry of biologically active molecules into cells that are maintained *in vitro* , such as in tissue culture. The
- 20   amphiphilic nature of the compounds of the invention enables them to associate with the lipids of cell membranes, other cell surface molecules, and tissue surfaces, and to fuse or to attach thereto. One type of structure that can be formed by amphiphiles is the liposome, a vesicle formed into a more or less spherical bilayer, that is stable in biological fluids and can entrap
- 25   biological molecules targeted for intracellular delivery. By fusing with cell membranes, such liposomal compositions permit biologically active molecules carried therewith to gain access to the interior of a cell through

one or more cell processes including endocytosis and pinocytosis.

However, unlike the case for many classes of amphiphiles or other lipid-like molecules that have been proposed for use in therapeutic compositions, the cationic amphiphiles of the invention need not form highly organized

- 5 vesicles in order to be effective, and in fact can assume (with the biologically active molecules to which they bind) a wide variety of loosely organized structures. Any of such structures can be present in pharmaceutical preparations of the invention and can contribute to the effectiveness thereof.

- 10 Biologically active molecules that can be provided intracellularly in therapeutic amounts using the amphiphiles of the invention include:

- (a) polynucleotides such as genomic DNA, cDNA, and mRNA that encode for therapeutically useful proteins as are known in the art,
- (b) ribosomal RNA;
- 15 (c) antisense polynucleotides, whether RNA or DNA, that are useful to inactivate transcription products of genes and which are useful, for example, as therapies to regulate the growth of malignant cells; and
- (d) ribozymes.

- In general, and owing to the potential for leakage of contents
- 20 therefrom, vesicles or other structures formed from numerous of the cationic amphiphiles are not preferred by those skilled in the art in order to deliver low molecular weight biologically active molecules. Although not a preferred embodiment of the present invention, it is nonetheless within the practice of the invention to deliver such low molecular weight molecules
- 25 intracellularly. Representative of the types of low molecular weight biologically active molecules that can be delivered include hormones and antibiotics.

Cationic amphiphile species of the invention may be blended so that two or more species thereof are used, in combination, to facilitate entry of biologically active molecules into target cells and/or into subcellular compartments thereof. Cationic amphiphiles of the invention can also be  
5 blended for such use with amphiphiles that are known in the art.

Dosages of the pharmaceutical compositions of the invention will vary, depending on factors such as half-life of the biologically-active molecule, potency of the biologically-active molecule, half-life of the amphiphile(s), any potential adverse effects of the amphiphile(s) or of  
10 degradation products thereof, the route of administration, the condition of the patient, and the like. Such factors are capable of determination by those skilled in the art.

A variety of methods of administration may be used to provide highly accurate dosages of the pharmaceutical compositions of the  
15 invention. Such preparations can be administered orally, parenterally, topically, transmucosally, or by injection of a preparation into a body cavity of the patient, or by using a sustained-release formulation containing a biodegradable material, or by onsite delivery using additional micelles, gels and liposomes. Nebulizing devices, powder inhalers, and aerosolized  
20 solutions are representative of methods that may be used to administer such preparations to the respiratory tract.

Additionally, the therapeutic compositions of the invention can in general be formulated with excipients (such as the carbohydrates lactose, trehalose, sucrose, mannitol, maltose or galactose) and may also be  
25 lyophilized (and then rehydrated) in the presence of such excipients prior to use. Conditions of optimized formulation for each amphiphile of the invention are capable of determination by those skilled in the



pharmaceutical art. By way of example, for spermidine cholesterol carbamate (amphiphile No. 53), it has been determined that use of sucrose is preferred over mannitol in order to prevent formation of amphiphile/DNA aggregates, particularly as the concentration of DNA is increased therein.

- 5 Addition of such excipients maintains the consistency of lyophilized pharmaceutical compositions during storage, and prevent difficulties such as aggregation, or insolubility, that may likely occur upon rehydration from the lyophilized state.

Accordingly, a principal aspect of the invention involves providing a  
10 composition that comprises a biologically active molecule (for example, a polynucleotide) and one or more cationic amphiphiles (including optionally one or more co-lipids), and then maintaining said composition in the presence of one or more excipients as aforementioned, said resultant composition being in liquid or solid (preferably lyophilized) form, so that:

- 15 (1) the therapeutic activity of the biologically active molecules is substantially preserved; (2) the transfection-enhancing nature of the amphiphile( or of amphiphile/ DNA complex) is maintained. Without being limited as to theory, it is believed that the excipients stabilize the interaction (complexes) of the amphiphile and biologically active molecule  
20 through one or more effects including:  
(1) minimizing interactions with container surfaces,  
(2) preventing irreversible aggregation of the complexes, and  
(3) maintaining amphiphile/DNA complexes in a chemically-stable state, i.e., preventing oxidation and/or hydrolysis.

- 25 Although the presence of excipients in the pharmaceutical compositions of the invention stabilizes the compositions and facilitates storage and manipulation thereof, it has also been determined that moderate

concentrations of numerous excipients may interfere with the transfection-enhancing capability of pharmaceutical formulations containing them. In this regard, an additional and valuable characteristic of the amphiphiles of the invention is that any such potentially adverse effect can be minimized

5 owing to the greatly enhanced in vivo activity of the amphiphiles of the invention in comparison with amphiphilic compounds known in the art. Without being limited as to theory, it is believed that osmotic stress ( at low total solute concentration) may contribute positively to the successful transfection of polynucleotides into cells in vivo . Such a stress may occur

10 when the pharmaceutical composition, provided in unbuffered water, contacts the target cells. Use of such otherwise preferred compositions may therefore be incompatible with treating target tissues that already are stressed, such as has damaged lung tissue of a cystic fibrosis patient. Accordingly, and using sucrose as an example, selection of concentrations of

15 this excipient that range from about 15 mM to about 200 mM provide a compromise between the goals of (1) stabilizing the pharmaceutical composition to storage and (2) minimizing any effects that high concentrations of solutes in the composition may have on transfection performance.

Selection of optimum concentrations of particular excipients for

20 particular formulations is subject to experimentation, but can be determined by those skilled in the art for each such formulation.

An additional aspect of the invention concerns the protonation state of the cationic amphiphiles of the invention prior to their contacting plasmid DNA in order to form a therapeutic composition. It is within the practice of

25 the invention to utilize fully protonated, partially protonated, or free base forms of the amphiphiles in order to form such therapeutic compositions. With respect to amphiphile No. 67 (spermine cholesterol carbamate), it has

been observed that when providing this amphiphile for a transfecting composition with DOPE (itself provided as a zwitterion), transgene expression was best for the free base, but decreased if the amphiphile was prepared as an acetate salt. Activity decreased step-wise through the mono  
5 and di acetate salts and was minimal for the tri-acetate salt. Under the circumstances described, the plasmid DNA provided for contacting with the amphiphile was prepared (without buffer) as a sodium salt in water.

### Methods of Syntheses

The following methods illustrate production of certain of the cationic amphiphiles of the invention. Those skilled in the art will recognize other methods to produce these compounds, and to produce also the other  
5 compounds of the invention.

### Group I amphiphiles

#### (A) N<sup>4</sup>-Spermidine cholesteryl carbamate

Spermidine cholesterol carbamate (Figure 1, No. 53) was synthesized according to the following procedure which is outlined in Figure 8.

#### 10 Synthesis of N<sup>1</sup>,N<sup>8</sup>-DiCBZ-N<sup>4</sup>-Spermidine Cholesterol Carbamate

N<sup>1</sup>,N<sup>8</sup>-dicarbobenzoxy spermidine (61% yield, m.p. 104 - 105° C) was prepared according to the procedure of S. K. Sharma, M. J. Miller, and S. M. Payne, J. Med. Chem., 1989, 32, 357-367. The N<sup>1</sup>,N<sup>8</sup>-dicarbobenzoxy spermidine (25 g, 60.5 mmol) and triethylamine (25 ml, 178  
15 mmol) were dissolved in 625 ml of anhydrous methylene chloride, cooled to 0 - 4°C and stirred under N<sub>2</sub>. Cholesteryl chloroformate (27.2 g, 60.6 mmol) was dissolved in 250 ml of methylene chloride and added to the reaction over a 20 minute period. A white precipitate formed upon addition. After the addition was complete, the reaction was stirred at 0 - 4°C for 10 minutes  
20 and then at room temperature for 1.5 hr. At this point, the white precipitate completely dissolved. The reaction was followed by TLC with hexane / ethyl acetate 6 / 4 as eluent (product R<sub>f</sub> = 0.25). To this reaction mixture was added 625 ml of methylene chloride and 625 ml of water. The layers were then allowed to separate. The organic layer was dried over MgSO<sub>4</sub>  
25 and filtered. The filtrate was concentrated *in vacuo* to give an oil. Vacuum drying was then carried out overnight. This crude product had a glue-like consistency. The crude product was purified by column chromatography (2

kg silica gel, eluent - hexane / ethyl acetate 6 / 4) to give 46.8 g of the 3- $\beta$ -[N<sup>4</sup>-(N<sup>1</sup>,N<sup>8</sup>-dicarbobenzoxy spermidine)carbamoyl] cholesterol (also described herein as N<sup>1</sup>,N<sup>8</sup>-diCBZ-N<sup>4</sup>-spermidine cholesterol carbamate) in 93% yield.

#### 5 Final Synthesis of Spermidine Cholesterol Carbamate

To 6.0 grams of 10% palladium on activated carbon under N<sub>2</sub> was added a solution of 30 grams of 3- $\beta$ -[N<sup>4</sup>-(N<sup>1</sup>,N<sup>8</sup>-dicarbobenzoxy spermidine)carbamoyl] cholesterol in 1 liter of ethanol, see Figure 13. The reaction mixture was purged with N<sub>2</sub> and stirred under H<sub>2</sub>  
10 (atmospheric pressure) for 18 hr. The mixture was again purged with N<sub>2</sub> and filtered through a 10 g bed of celite. The filter cake was washed with 2 liters of 10% triethylamine in ethanol and the combined filtrates were concentrated *in vacuo* to a gel. The product was then dried under vacuum overnight to a sticky solid. This crude product was purified by column  
15 chromatography (2 kg of silica gel, eluent - 4 L of chloroform / methanol 95 / 5 followed by 30 L of chloroform / methanol / iso-propylamine 95 / 5 / 5, R<sub>f</sub> = 0.24) to obtain 13.1 g of the desired spermidine cholesterol carbamate in 64% yield. HPLC (C-18 reversed phase column, linear gradient elution profile - methanol / iso-propanol / water / trifluoroacetic acid 60 / 20 / 20  
20 / 0.1 to methanol / iso-propanol / trifluoroacetic acid 70 / 30 / 0.1 to methanol / iso-propanol / chloroform / trifluoroacetic acid 60 / 20 / 20 / 0.1) analysis of this material showed it to be 99.2% pure with the 7-dehydrocholesterol analog present at a level of 0.8%.

In connection with this example and those that follow, it is noted that  
25 all TLC plates were visualized with phosphomolybdic acid.

(B) N<sup>4</sup>-Spermine cholesteryl carbamate

Spermine cholesterol carbamate (Figure 1, No. 67) was prepared according to the following procedure which is outlined in Figure 9.

N<sup>1</sup>N<sup>12</sup>-diCBZ-spermine

- 5       Benzylchloroformate (1.76g, 1.5 ml, 10.36 mmol) was dissolved in methylene chloride (5 ml) and placed in a three neck flask under a nitrogen atmosphere. Imidazole (1.4 g, 20.6 mmol) was dissolved in methylene chloride (20 ml) and placed in an addition funnel. The three neck flask was cooled to 0°C and the imidazole solution was added gradually over 20 min.
- 10   The mixture was stirred at room temperature for 1 hour and then methylene chloride (25 mL) and citric acid (10%, 25 ml) were added. The layers were separated and the organic fraction was washed with citric acid (10%, 25 ml). The organic component was dried over magnesium sulfate and concentrated in vacuo. The residue was dried under high vacuum for 1 hour
- 15   at ambient temperature.

- To the residue was added dimethylaminopyridine (35 mg), methylene chloride (25 ml) and the mixture was cooled to 0°C, under a nitrogen atmosphere. To an addition funnel was added a solution of spermine (1g, 4.94 mmol) in methylene chloride (25 ml). The spermine
- 20   solution was added gradually over 15 min. The reaction mixture was stirred overnight at ambient temperature and then concentrated in vacuo. The residue was dissolved in ethyl acetate (80 ml) and washed three times with water (15 ml). The organics were dried over magnesium sulfate, filtered and concentrated in vacuo to give a crude white solid. The material was purified
- 25   by flash chromatography (65g silica gel, 100:100:10 CHCl<sub>3</sub>: MeOH: NH<sub>4</sub>OH, product R<sub>f</sub>=0.33), to give after drying under high vacuum 1.01g (2.146 mmol, 43 % yield) of product.

N<sup>1</sup>N<sup>12</sup>-diCBZ-N<sup>4</sup>-spermine cholesteryl carbamate

Cholesteryl chloroformate (964 mg, 2.15 mmol) was dissolved in chloroform (10 ml) and added dropwise to a cooled (0°C) solution of N<sup>1</sup>N<sup>12</sup>-diCBZ spermine (1.01g, 2.15 mmol), triethylamine (1 ml) in  
5 chloroform (10 ml). The reaction was allowed to warm to room temperature and stirred for 2 hours. To the reaction solution was added water (25 ml) and chloroform (25 ml). The layers were separated and the organic fraction dried over magnesium sulfate. The solution was concentrated in vacuo to give a crude material that was purified by flash chromatography (68g silica  
10 gel, MeOH / CHCl<sub>3</sub> 1/4, product R<sub>f</sub> = 0.36) to give 1.23 g (1.39 mmol, 65% yield) of product.

final synthesis of N<sup>4</sup>-Spermine Cholesteryl Carbamate

N<sup>1</sup>N<sup>12</sup>-diCBZ-N<sup>4</sup>-spermine cholesteryl carbamate (262 mg, 0.300 mmol) was dissolved in 5 ml of acetic acid and 45 mg of 10% Pd on C was  
15 added. The solution was purged with nitrogen and stirred under hydrogen at atmospheric pressure. The hydrogenolysis was allowed to proceed for 7 hours. The reaction mixture was filtered and the catalyst was washed with 40 ml of ethyl acetate / acetic acid 9 / 1 and the filtrate will be concentrated  
in *vacuo* to give a residue. The crude product was dissolved in 35 mL of 1N  
20 NaOH and extracted three times with 40 ml of chloroform / methanol 9 / 1. The combined organic fractions were washed with 20 mL of water and dried over Na<sub>2</sub>SO<sub>4</sub>. The solution was filtered, concentrated in *vacuo* and dried under vacuum to give 125 mg of the desired product in 67% yield.

In connection with the above procedure, it is noted that the  
25 hydrogenolysis should be carried out under acidic conditions, in order to minimize the poisoning of the catalyst.

Urea analogs - such as spermine or spermidine cholestamine urea - can be prepared by a sequence of reactions well known to those versed in the art of organic synthesis. For example an amine can be treated with an equal molar amount of carbonyldiimidazole followed by the addition of a second amine to give the desired urea.

(C) N,N Bis (3-aminopropyl)-O-cholesteryl-3-carbamate

N,N Bis (3-aminopropyl)-O-cholesteryl-3-carbamate (Figure 1, No. 69) was prepared according to the following procedure.

Bis (3-CBZ aminopropyl) amine was prepared using the method described above for N<sup>1</sup>,N<sup>12</sup>-diCBZ-spermine, except that N-(3-aminopropyl)1,3-propanediamine was substituted for spermine as reactant. The pure product was isolated in 34 % yield by silica gel flash chromatography using as solvent CHCl<sub>3</sub>/ MeOH/ NH<sub>4</sub>OH 80/20/0.5.

The Bis (3-CBZ aminopropyl) amine so prepared was then reacted with cholesteryl chloroformate according to the method described above for the synthesis of N<sup>1</sup>, N<sup>8</sup>-DiCBZ -N<sup>4</sup>-spermidine cholesteryl carbamate. The pure product (N,N Bis ( 3-CBZ aminopropyl)-O-cholesteryl-3-carbamate) was obtained in 73% yield.

Synthesis of N,N Bis(3-aminopropyl)-O-cholesteryl-3-carbamate was completed by hydrogenolysis of the CBZ groups from N,N Bis(3-CBZ aminopropyl)-O-cholesteryl-3-carbamate following the procedure described above in relation to the synthesis of N<sup>4</sup>-spermidine cholesteryl carbamate. The product was obtained in 23% yield without silica gel chromatography purification.

(D) N,N Bis (6-aminoethyl)-O-cholesteryl-3-carbamate.

N,N Bis (6-aminoethyl)-O-cholesteryl-3-carbamate (Figure 1, No. 70) was prepared according to the following procedure.



First, Bis (6-CBZ aminoethyl) amine was prepared using the method described above for N<sup>1</sup>,N<sup>12</sup> -diCBZ-spermine, except that Bis(hexamethylene)triamine was substituted for spermine as reactant. Pure product was isolated in 24% yield by recrystallization from toluene.

5        Bis (6-CBZ aminoethyl) amine was then reacted with cholesteryl chloroformate according to the method described above for the synthesis of N<sup>1</sup>,N<sup>8</sup>-DiCBZ -N<sup>4</sup>-spermidine cholesteryl carbamate. Product N,N Bis(6-CBZ aminoethyl)-O-cholesteryl-3-carbamate was isolated in 40% yield by silica gel flash chromatography using hexanes/ethyl acetate 7/3 .

10                    (E) Lysine 3-N- dihydrocholesteryl carbamate

Lysine 3-N- dihydrocholesteryl carbamate (Figure 1, panel C) was prepared according to the following procedure.

To a solution of dihydrocholesterol (5.0 g, 12.9 mmol, Aldrich), phthalimide (2.0 g, 13.6 mmol, Aldrich), and triphenylphosphine (3.8 g, 13.6  
15    mmol, Aldrich) in THF (20 ml, Aldrich) stirred at 0° C under a nitrogen atmosphere was added dropwise diethylazodicarboxylate (2.3 ml, 14.5 mmol, Aldrich). Upon the completion of addition the reaction mixture was allowed to warm to ambient temperature and stirred overnight. The reaction mixture was concentrated *in vacuo* to a residue. This residue was  
20    dissolved in 50 ml hexane / ethyl acetate 95 / 5 and a precipitate formed. The mixture was filtered. The filtrate was concentrated to dryness *in vacuo*, dissolved in 25 ml of hexane / ethyl acetate 95 / 5 and chromatographed on 200 g silica gel (eluent 2 L hexane / ethyl acetate 95 / 5 then 1 L hexane / ethyl acetate 90 / 10). A 76% yield of the desired 3-phthalimidocholestane  
25    (5.43 g) was obtained.

The 3-phthalimidocholestane (5.40 g, 9.75 mmol) was dissolved in 60 mL of methanol and anhydrous hydrazine (3.1 ml, 99 mmol) was added.

The reaction mixture was stirred and heated at reflux under a nitrogen atmosphere for 4 hr. This mixture was then cooled to room temperature, 3.1 mL of concentrated HCl was added and the resulting mixture was heated at reflux overnight. Upon cooling to ambient temperature, 100 ml of diethyl ether and 50 ml of 1 N NaOH were added (final pH of 10.1) and the layers were separated. The aqueous layer was extracted with 50 ml of diethyl ether and the combined organic fractions were filtered. The filtrate was concentrated *in vacuo* and the residue was purified by silica gel chromatography (chloroform / methanol 90 / 10) to give 2.24 g of 3-aminocholestane in 59 % yield.

L-N $\alpha$ ,N $\epsilon$ -diBOClysine N-hydroxysuccinimide ester (286 mg, 0.644 mmol, Sigma) and 3-aminocholestane (250 mg, 0.644 mmol) were dissolved in 5 mL of methylene chloride, 0.1 mL of triethylamine was added and the resulting solution was stirred under a nitrogen atmosphere at ambient temperature overnight. To the reaction mixture was added 10 mL of water and 25 mL of methylene chloride and the layers were separated. The aqueous layer was extracted with 25 mL of methylene chloride and the combined organic fractions were dried over MgSO<sub>4</sub> and filtered. The filtrate was concentrated *in vacuo* and the residue was purified by chromatography on 25 g of silica gel (eluent - hexane / ethyl acetate 6 / 4, sample applied in hexane / ethyl acetate 9 / 1). The purified material was dissolved in 25 mL of chloroform and HCl gas was bubbled through the solution for 2 hr. followed by nitrogen for 10 min. The solution was concentrated *in vacuo* to give 299 mg of the desired product in 79% yield as the dihydrochloride salt.

(F) N<sup>1</sup>N<sup>8</sup>-Bis(3-aminopropyl)-N<sup>4</sup>-spermidine cholesteryl carbamate  
N<sup>1</sup>N<sup>8</sup>-Bis(3-aminopropyl)-N<sup>4</sup>-spermidine cholesteryl carbamate

(Figure 1, No. 75) was prepared according to the following procedure.

N<sup>4</sup>-Spermidine cholesteryl carbamate (1.14g, 2.04 mmol) was dissolved  
5 in MeOH (5 mL). Freshly distilled acrylonitrile (0.28 mL, 4.29 mmol) was  
added and the solution was stirred at room temperature for 18 h. The  
solvent was concentrated *in vacuo* to give an oil. Vacuum drying was then  
carried out overnight. The crude product was purified by column  
chromatography (125 g silica gel, eluent - CHCl<sub>3</sub> MeOH 1/9) to give 1.15 g  
10 (85 %) of the N<sup>1</sup>N<sup>8</sup>-Bis (cyanoethyl) N<sup>4</sup>-Spermidine cholesteryl carbamate.

Raney Nickel 50% slurry (1.2 g, Aldrich) was placed in a Parr Bomb with  
1M NaOH in 95% EtOH (50 mL). The N<sup>1</sup>N<sup>8</sup>-Bis (cyanoethyl) N<sup>4</sup>-  
Spermidine cholesteryl carbamate. was dissolved in EtOH (35 mL) and  
added to the bomb. The vesicle was evacuated and placed under Argon  
15 pressure (80-100 psi), three times and then evacuated and placed under  
Hydrogen pressure (100 psi), three times. The reaction was stirred under  
hydrogen pressure (100 psi) at room temperature for 72h. The vesicle was  
evacuated and placed under argon pressure. The catalyst was removed by  
filtration. The filtrate was concentrated *in vacuo* . The resulting oil was  
20 dissolved in 2:1 CH<sub>2</sub>Cl<sub>2</sub>: MeOH (100 mL) and washed with H<sub>2</sub>O (35 and 25  
mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and filtered. The filtrate was  
concentrated *in vacuo* and the residue was purified by chromatography on  
100 g of silica gel (eluent - CHCl<sub>3</sub>/MeOH/conc. NH<sub>4</sub>OH 40/25/10, sample  
applied in CHCl<sub>3</sub>/MeOH 40/25). The purified material was concentrated *in*  
25 *vacuo* with iPrOH (3 X 50 mL) and CH<sub>2</sub>Cl<sub>2</sub>(3X50 mL) and then vacuum  
dried to give 986 mg (85%) of N<sup>1</sup>N<sup>8</sup>-Bis(3-aminopropyl)-N<sup>4</sup>-spermidine  
cholesteryl carbamate.

(G) N(N<sup>4</sup>-3-aminopropyl-spermidine) cholesteryl carbamate

N(N<sup>4</sup>-3-aminopropyl-spermidine) cholesteryl carbamate (Figure 1, No. 78) was prepared as follows:

N<sup>1</sup>, N<sup>8</sup>-dicarbobenzoxy spermidine (1.0 g, 2.4 mmol) was dissolved in MeOH (10 mL). Freshly distilled acrylonitrile (0.3 mL, 4.5 mmol) was added and the reaction was stirred at room temperature for 18 h. The solvent was concentrated *in vacuo* to give an oil. The crude product was purified by column chromatography (100 g silica gel, eluent - CHCl<sub>3</sub>/MeOH 1/19) to give 1.10 g (97 %) of N<sup>4</sup>-2-Cyanoethyl-N<sup>1</sup>, N<sup>8</sup> - dicarbobenzoxy spermidine.

The N<sup>4</sup>-2-Cyanoethyl-N<sup>1</sup>, N<sup>8</sup>-dicarbobenzoxy spermidine (0.5 g, 1.07 mmol) was dissolved in MeOH (5 mL) and CoCl<sub>2</sub> (280 mg, 2.15 mmol, Aldrich) was added. The blue solution was cooled in an ice bath and NaBH<sub>4</sub> (405 mg, 10.7 mmol, Aldrich) was added in portions over 15 min. The resulting black solution was stirred at room temperature for 1 h. The black solution turned blue over this period. To the reaction was added CH<sub>2</sub>Cl<sub>2</sub>/MeOH 2/1 (30 mL). A black ppt formed. To this was added H<sub>2</sub>O (20 mL) and the mixture was filtered. The resulting layers were separated and the organic layer dried with MgSO<sub>4</sub>. The drying agent was filtered and the filtrate concentrated *in vacuo* to give an oil. The crude product was purified by column chromatography (50 g silica gel, eluent - CHCl<sub>3</sub>/MeOH/conc NH<sub>4</sub>OH 100/100/5) to give 309 mg (62 %) of the N<sup>4</sup>-3-aminopropyl-N<sup>1</sup>, N<sup>8</sup> - dicarbobenzoxy spermidine.

To the N<sup>4</sup>-3-aminopropyl-N<sup>1</sup>, N<sup>8</sup> - dicarbobenzoxy spermidine (300 mg, 0.66 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> was added Et<sub>3</sub>N under N<sub>2</sub>. Cholesteryl chloro formate (326 mg, 0.726 mmol, Aldrich) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and added to the reaction dropwise. The mixture was stirred for 2h at room temperature. After adding CH<sub>2</sub>Cl<sub>2</sub> (25 mL) and H<sub>2</sub>O (10 mL), the layers

were separated. The organic layer was dried with  $\text{MgSO}_4$  and filtered. The filtrate was concentrated *in vacuo* to give 640 mg of crude product. The residue was purified by chromatography on 80 g of silica gel (eluent -  $\text{CHCl}_3$  /  $\text{MeOH}$  90 / 10, sample applied in  $\text{CHCl}_3$  /  $\text{MeOH}$  90/10). The  
 5 purified material was concentrated *in vacuo* and then vacuum dried to give 329 mg (57%) of N-(N<sup>4</sup>-3-aminopropyl-N<sup>1</sup>, N<sup>8</sup> - dicarbobenzoxy spermidine) cholesteryl carbamate.

To 10% Pd on carbon (65 mg, Aldrich) was added a solution of N-(N<sup>4</sup>-3-aminopropyl-N<sup>1</sup>, N<sup>8</sup> - dicarbobenzoxy spermidine) cholesteryl carbamate  
 10 (300 mg) in acetic acid (25 mL). The reaction was placed under  $\text{H}_2$  and stirred at room temperature overnight. After being placed under  $\text{N}_2$ , the reaction was filtered. The catalyst was washed with 10 % acetic acid in EtOAc (50 mL). The filtrate was concentrated *in vacuo* to give an oil. The oil was dissolved in 2/1  $\text{CH}_2\text{Cl}_2$ /MeOH (35 mL) and washed with 1 M NaOH  
 15 (15 mL). The organic layer was dried with  $\text{MgSO}_4$  and filtered. The filtrate was concentrated *in vacuo* and vacuum dried to give 196 mg (93%) of N-(N<sup>4</sup>-3-aminopropyl spermidine) cholesteryl carbamate.

(H) N-[N<sup>1</sup>,N<sup>4</sup>,N<sup>8</sup>-Tris (3-aminopropyl) spermidine] cholesteryl carbamate

20 N-[N<sup>1</sup>,N<sup>4</sup>,N<sup>8</sup>-Tris (3-aminopropyl) spermidine] cholesteryl carbamate (Figure 1, No. 96) was prepared by reacting N-(N<sup>4</sup>-3-aminopropyl spermidine) cholesteryl carbamate with acrylonitrile (90% yield) and subsequent reduction of the di adduct with Raney nickel (75 % yield) as described for the preparation of N<sup>1</sup>,N<sup>8</sup>Bis(3-aminopropyl)-N<sup>4</sup>-  
 25 spermidine cholesteryl carbamate.

(I) N,N-Bis(4-aminobutyl) cholesteryl carbamate

N,N-Bis(4-aminobutyl) cholesteryl carbamate (Figure 1, No. 82) was prepared as follows.

To a mixture of Benzylamine (2.0 g, 18.6 mmol, Aldrich), Na<sub>2</sub>CO<sub>3</sub> (4.4g, 42 mmol) and KI (1.4 g, 9.5 mmol) in n-butanol (50 mL) was added 4-Chlorobutyronitrile (4.0 mL, 95 mmol) under nitrogen. The reaction was stirred at reflux of 48 h under nitrogen. After cooling to room temperature, diethyl ether (50 mL) was added and the precipitate filtered off. The filtrate was concentrated *in vacuo* to an oil. Toluene (100 mL) was added and the solution was concentrated *in vacuo*. Chloroform (100 mL) was added and again the solution was concentrated *in vacuo* and then vacuum dried for 18 h. The resulting oil was dissolved in Chloroform (100 mL) filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (250 g silica gel, eluent - hexanes/EtOAc 60/40) to give 3.75g (97 %) of N,N-Bis (3-cyanopropyl) benzylamine.

The N,N-Bis (3-cyanopropyl) benzylamine (3.7 g, 17.8 mmol) was dissolved in EtOH (150 mL) and Acetic acid (4 mL) was added. This solution was added to 10% Pd on carbon (400 mg) under N<sub>2</sub>. The mixture was placed under H<sub>2</sub> and the reaction stirred for 18 h at room temperature. The reaction was placed under N<sub>2</sub>. The catalyst was filtered off and washed with EtOH (150 mL). The filtrate was concentrated *in vacuo*, chloroform (50 mL) was added and again concentrated *in vacuo*. The resulting oil was vacuum dried for 0.5 h and used directly in the next reaction. To this oil dissolved in CH<sub>2</sub>Cl<sub>2</sub> (100mL) was added Et<sub>3</sub>N (5 mL, 35 mmol) under N<sub>2</sub> and the solution cooled in an ice bath. Cholesteryl chloro formate (6.2 g, 13.87 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (100 mL) and this solution was added to the reaction dropwise over 10 min. The cooling bath was removed and the

reaction stirred at room temperature for 18 h under N<sub>2</sub>. CH<sub>2</sub>Cl<sub>2</sub> (100 mL) and H<sub>2</sub>O (100 mL) was added and the resulting layers were separated. The organic layer was dried with MgSO<sub>4</sub> and filtered. The filtrate was concentrated *in vacuo* and vacuum dried for 1 h. The crude product was  
5 purified by column chromatography (600 g silica gel, eluent - hexanes/EtOAc 60/40) to give 1.05g (10 %) of N,N-Bis (3-cyanopropyl) cholesteryl carbamate.

Raney Nickel 50% slurry (1.2 g) was placed in a Parr Bomb with 1M NaOH in 95% EtOH (50 mL). The N,N-Bis (3-cyanopropyl) cholesteryl  
10 carbamate (1.0 g, 1.77 mmol) was dissolved in EtOH (100 mL) and added to the bomb. The vesicle was evacuated and placed under Argon pressure (80-100 psi), three times and then evacuated and placed under Hydrogen pressure (100 psi), three times. The reaction was stirred under hydrogen pressure (100 psi) at room temperature for four days. The vesicle was  
15 evacuated and placed under argon pressure. The catalyst was removed by filtration. The filtrate was concentrated *in vacuo*. The resulting oil was dissolved in 2:1 CH<sub>2</sub>Cl<sub>2</sub>: MeOH (250 mL) and washed twice with H<sub>2</sub>O (75 and 50 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and filtered. The filtrate was concentrated *in vacuo* and the residue was purified by  
20 chromatography on 110 g of silica gel (eluent - CHCl<sub>3</sub>/MeOH/iPrNH<sub>2</sub> 95/5/5, sample applied in CHCl<sub>3</sub>/MeOH 95/5). The purified material was concentrated *in vacuo* and then vacuum dried to give 900 mg (85%) of N,N-Bis(4-aminobutyl) cholesteryl carbamate.

(I) N,N-Bis(N'-3-aminopropyl-4-aminobutyl) cholesteryl carbamate  
25 N,N-Bis(N'-3-aminopropyl-4-aminobutyl) cholesteryl carbamate (Figure 1, No. 83) was prepared by reacting N,N-Bis(4-aminobutyl) cholesteryl carbamate with acrylonitrile (82% yield) and subsequent

reduction of the di acrylonitrile adduct with Raney nickel (81 % yield) as described for the preparation of N<sup>1</sup>,N<sup>8</sup>-Bis(3-aminopropyl)-N<sup>4</sup>-spermidine cholesteryl carbamate.

(K) N<sup>4</sup> Spermidine cholesteryl carboxamide

5 N<sup>4</sup> Spermidine cholesteryl carboxamide ( Figure 1, No. 90) was prepared as follows.

A solution of cholesteryl chloride (5.0 g, 12.3 mmol) in THF (50 mL) was added dropwise over 0.5 h under reflux to Magnesium turnings (390 mg) in THF (25 mL). Initially a pinch of Iodine and three drops of  
10 Iodomethane were added to initiate the reaction. After refluxing for 3 h. the reaction was cooled to room temperature. This mixture was poured onto Dry ice (10 g) and then stirred for 1h. This solution was cooled in an ice bath and added to ice cold 1 M H<sub>2</sub>SO<sub>4</sub> (100 mL). After stirring for 5 min., sodium chloride (1 g) and diethyl ether (100 mL) was added. The layers were  
15 separated and the aqueous layer was extracted with diethyl ether (100 mL). The combined organic layers were washed with a solution of Sodium thiosulfate pentahydrate(120 mg) in H<sub>2</sub>O (30 mL). The organic layer was concentrated *in vacuo* and vacuum dried for 18 h. The crude solid was titrated with hexanes (25 mL). After filtration the solid was washed with ice  
20 cold hexanes (10 mL). The solid was vacuum dried for 1h. The cholesteryl carboxylic acid obtained (3.0 g, 59 %) was ca. 90 % pure and used without further purification.

Cholesteryl carboxylic acid (500 mg, 1.2 mmol) and N-hydroxysuccinimide (140 mg, 1.2 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub>. To this  
25 solution was added Dicyclohexylcarbodiimide (275 mg, 1.32 mmol) was added and the reaction was stirred under N<sub>2</sub> for 2h. N<sup>1</sup>,N<sup>8</sup>-dicarbobenzoxy spermidine (474 mg, 1.2 mmol) and Et<sub>3</sub>N (1.0 mL, 7.1 mmol)



was added and the reaction was stirred under N<sub>2</sub> for 72 h. The reaction was filtered and the precipitate was washed with CH<sub>2</sub>Cl<sub>2</sub> (50 mL). The filtrate was washed with H<sub>2</sub>O (25 mL). The separated organic layer was dried over MgSO<sub>4</sub> and filtered. The filtrate was concentrated *in vacuo* and the residue  
5 was purified by chromatography on 150 g of silica gel (eluent - hexanes / EtOAc 1/ 1). The purified material was concentrated *in vacuo* and then vacuum dried to give 680 mg (70%) of N<sup>1</sup>,N<sup>8</sup>-dicarbobenzoxy-N<sup>4</sup>-spermidine cholesteryl carboxamide.

The carbobenzoxy group were removed from N<sup>1</sup>,N<sup>8</sup>-  
10 dicarbobenzoxy-N<sup>4</sup>-spermidine cholesteryl carboxamide as described in the preparation of N<sup>4</sup>-spermidine cholesteryl carbamate. The purified product, N<sup>4</sup> Spermidine cholesteryl carboxamide, was obtained in 53 % yield.

#### Group II Amphiphiles

(A) N<sup>1</sup>,N<sup>8</sup>-Bis(Arginine carboxamide)-N<sup>4</sup>-spermidine cholesteryl  
15 carbamate

N<sup>1</sup>,N<sup>8</sup>-Bis(Arginine carboxamide)-N<sup>4</sup>-spermidine cholesteryl  
carbamate (Figure 5, No. 95) was prepared as follows.

To N<sup>(a)</sup>,N<sup>(e)</sup>,N<sup>(e)</sup> (alpha, epsilon, epsilon) -tricarobenzoxyArginine  
in CH<sub>2</sub>Cl<sub>2</sub> (25 mL) was added N-hydroxysuccinimide (100 mg, 0.89 mmol)  
20 and dicyclohexylcarbodiimide (240 mg, 0.89 mmol). The mixture was stirred  
under N<sub>2</sub> at room temperature for 2.5 hours. N<sup>4</sup>- Spermidine Cholesteryl  
Carbamate (250 mg, 0.448 mmol) and Et<sub>3</sub>N ( 0.25 mL, 1.8 mmol) was added  
and the reaction stirred at room temperature under N<sub>2</sub> for 72 h. The  
reaction was filtered and the precipitate was washed with CH<sub>2</sub>Cl<sub>2</sub> (20 mL).  
25 The filtrate was washed with H<sub>2</sub>O (20 mL). The separated organic layer was  
dried over MgSO<sub>4</sub> and filtered. The filtrate was concentrated *in vacuo* and  
the residue was purified by chromatography on 70 g of silica gel (eluent -

CHCl<sub>3</sub> / MeOH 95/ 5). The purified material was concentrated *in vacuo* and then vacuum dried to give 533 mg (71%) of N<sup>1</sup>, N<sup>8</sup>-Bis (N(a) N(e) N(e)-tricarbobenzoxoArginine carboxamide)-N<sup>4</sup>-spermidine cholesteryl carbamate.

- 5 The carbobenzoxo group were removed from N<sup>1</sup>, N<sup>8</sup>-Bis (N(a) N(e) N(e)-tricarbobenzoxoArginine carboxamide)-N<sup>4</sup>-spermidine cholesteryl carbamate as described in the preparation of N-(N<sup>4</sup>-3-aminopropylspermidine) cholesteryl carbamate. The product, N<sup>1</sup>, N<sup>8</sup>-Bis(Arginine carboxamide)-N<sup>4</sup>-spermidine cholesteryl carbamate was
- 10 obtained in 27 % yield.

### Group III Amphiphiles

#### (A) N,N-Dioctadecyllysineamide

- N,N- dioctadecyllysineamide( Figure 6, No.73) was prepared according to the following procedure. N,N-dioctadecylamine (1.35 g, 2.58
- 15 mmol, Fluka) and L-N $\alpha$ ,N $\epsilon$ -diBOClysine N-hydroxysuccinimide ester ( 1.00 g, 2.58 mmol, Sigma) were combined in 15 ml of methylene chloride and 2 ml triethylamine was added. The reaction mixture was heated briefly to effect complete dissolution and then stirred at ambient temperature overnight. Water (20 ml) and methylene chloride (50 ml) were added to the
  - 20 reaction mixture and the layers were separated. The aqueous fraction was extracted a second time with 50 ml methylene chloride. The combined organic fractions were dried over MgSO<sub>4</sub>, filtered and concentrated *in vacuo*. The residue was purified by column chromatography (150 g silica gel , eluent - hexane/ethyl acetate 8/2). The purified material, N,N-dioctadecyl-
  - 25 N $\alpha$ ,N $\epsilon$ -diBOC lysineamide(1.59 g) was dissolved in 25 ml of chloroform and stirred for 2 hr. while HCl gas was bubbled through the solution. This solution was purged with N<sub>2</sub> gas and concentrated *in vacuo*. N,N

-dioctadecyllysineamide (1.34 g) was obtained in 68% yield as the di HCl salt.

(B) N<sup>1</sup>N<sup>1</sup>-Dioctadecyl-1,2,6-triaminohexane

N<sup>1</sup>N<sup>1</sup>-Dioctadecyl-1,2,6-triaminohexane (Figure 6, No. 47) was  
5 prepared as follows. To N,N-Dioctadecyl-N $\alpha$ ,N $\epsilon$ -diBOClysineamide (760  
mg, 0.823 mmol) in 30 ml anhydrous THF stirred at ambient temperature  
was added LiAlH<sub>4</sub> (185 mg, 4.87 mmol) in portions. The reaction mixture  
was stirred at ambient temperature overnight under a nitrogen atmosphere.  
The reaction was quenched by the dropwise addition of 2 ml water and the  
10 resulting solution was concentrated *in vacuo*. To this residue was added in  
order 10 mL of 1 M HCl, 50 ml of methylene chloride, and 10 ml of 1M  
NaOH (final pH 10). The layers were separated and the aqueous fraction  
was extracted a second time with 50 ml of methylene chloride. The  
combined organic layers were dried over MgSO<sub>4</sub> and filtered. The filter  
15 cake was washed with 50 ml of methylene chloride. The combined filtrates  
were concentrated *in vacuo* to give 700 mg of crude product. The crude  
product was purified by column chromatography (80 g silica gel, eluent -  
hexane/ethyl acetate 7/3). The fractions containing the purified product  
were combined and concentrated *in vacuo* to obtain 490 mg of the product  
20 protected as the diBOC derivative. To 200 mg of this diBOC derivative was  
added 4 ml of chloroform and 1 ml of TFA. The resulting reaction mixture  
was stirred at ambient temperature for 2 hr and concentrated *in vacuo*. The  
residue was dissolved in 25 ml of water and 25 mL of methylene chloride  
and adjusted to pH 10 with approximately 2 ml of concentrated ammonium  
25 hydroxide. The layers were separated and the aqueous layer was extracted  
a second time with 25 ml of methylene chloride. The organic fractions were  
combined, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo*. The resulting

residue was dissolved in 10 ml of diethyl ether, HCl gas was bubbled through the solution for 2 minutes and the solution was cooled at 4° C overnight. The precipitated product was collected by filtration, washed with cold (4° C) diethyl ether, and dried under vacuum to obtain 160 mg of the desired product in 67% yield.

#### Group IV Amphiphiles

##### (A) 1-(N<sup>4</sup>-spermine)-2,3-dilaurylglycerol carbamate

1-(N<sup>4</sup>-spermine)-2,3-dilaurylglycerol carbamate (Figure 7, No. 89) was prepared as follows. A solution of 3-benzyloxy-1,2-propanediol (1.00 g, 5.49 mmol) in THF (20 mL) was added to a suspension of sodium hydride (60% w/w in oil, 550 mg, 13.725 mmol) in THF (30 mL) and allowed to reflux overnight under dry nitrogen. A solution of dodecyl methane sulfonate (3.39 g, 12.078 mmol) in THF (20 mL) was added and the reaction was refluxed for another two days. After cooling to room temperature the reaction was filtered through a bed of Celite, rinsing with THF. The filtrate was reduced *in vacuo* to a yellow oil which was redissolved in diethyl ether (100 mL). The ether solution was washed with 0.1 N NaOH (30 mL) and dH<sub>2</sub>O (2 x 30 mL). The organic layer was dried over magnesium sulfate, filtered and reduced *in vacuo* to a red-brown oil. The crude material was purified by flash column chromatography (300 g silica gel) eluting with 3% ethyl acetate/ hexanes. The desired product was isolated as a pale yellow oil and characterized by <sup>1</sup>H NMR as 3-OBn-1,2-dilaurylglycerol (1.70 g, 60%). 3-OBn-1,2-dilaurylglycerol (1.70 g, 3.28 mmol) in ethanol (100 mL) was stirred with 10% Pd/C (250 mg, 15 wt%) under a hydrogen atmosphere for 24 hours. The reaction was flushed with nitrogen and filtered through Celite, rinsing with ethanol, to remove the catalyst. The filtrate was reduced *in vacuo* to a solid. The crude material was purified by flash column

chromatography (140 g silica gel) eluting with 10% ethyl acetate/ hexanes. The desired product was isolated as a white solid and characterized by  $^1\text{H}$  NMR as 1,2-dilaurylglycerol (1.23g, 88%).

A 1.93 M solution of phosgene in toluene (0.77 mL, 1.49 mmol) was  
5 added to a solution of 1,2-dilaurylglycerol (580 mg, 1.35 mmol) and N,N-diisopropylethylamine (0.26 mL, 1.49 mmol) in methylene chloride (10 mL) and stirred overnight. A solution of N<sup>1</sup>,N<sup>12</sup>-di-CBz-spermine•2HCl (734 mg, 1.35 mmol) in 60: 25: 4 chloroform/ methanol/ water (80 mL) was added. After 3 hours another equivalent of N,N-diisopropylethylamine  
10 (0.26 mL, 1.49 mmol) was added. An additional 0.5 equivalents of N,N-diisopropylethylamine (0.13 mL, 0.75 mmol) was added three hours later and the reaction was allowed to stir overnight under nitrogen at ambient temperature. The reaction was washed with 1M NaOH (20 mL) and dH<sub>2</sub>O (15 mL). The organic layer was separated, dried over magnesium sulfate,  
15 filtered and reduced *in vacuo* to a white solid The crude material was purified by flash column chromatography (125 g silica gel) eluting with 90: 10: 0.5 chloroform/ methanol/ ammonium hydroxide. The desired product was isolated as an oil and characterized by  $^1\text{H}$  NMR as 1-(N<sup>4</sup>-(N<sup>1</sup>,N<sup>12</sup>-di-CBz-spermine))-2,3-dilaurylglycerol carbamate (188 mg, 15%).

20 The 1-(N<sup>4</sup>-(N<sup>1</sup>,N<sup>12</sup>-di-CBz-spermine))-2,3-dilaurylglycerol carbamate (188 mg, 0.203 mmol) was dissolved in glacial acetic acid (10 mL) and stirred with 10% Pd/C (45 mg, 24 wt %) under a hydrogen atmosphere for 5 hours. The catalyst was removed by vacuum filtration rinsing with 10% acetic acid/ ethyl acetate (10 mL) The filtrate was reduced to an oil by rotary  
25 evaporation. The resulting oil was dissolved in 10% methanol/ chloroform (85 mL) and was washed with 1M NaOH (15 mL) and dH<sub>2</sub>O (10 mL). The organic layer was separated, dried over magnesium sulfate, filtered and

reduced *in vacuo* to an oil. The product was characterized by  $^1\text{H}$  NMR as 1-(N<sup>4</sup>-spermine)-2,3-dilaurylglycerol carbamate (125 mg, 94%).

Other amphiphiles of the invention may be prepared according to procedures that are within the knowledge of those skilled in art.

### Examples

The following Examples are representative of the practice of the invention.

#### Example 1 - Cell Transfection Assay

5        Separate 3.35  $\mu$ mole samples of spermidine cholesterol carbamate (amphiphile No. 53) and the neutral lipid dioleoylphosphatidylethanolamine ("DOPE") were each dissolved in chloroform as stock preparations. Following combination of the solutions, a thin film was produced by removing chloroform from the mixture by  
10    evaporation under reduced pressure (20 mm Hg). The film was further dried under vacuum (1 mm Hg) for 24 hours. As aforementioned, some of the amphiphiles of the invention participate in transacylation reactions with co-lipids such as DOPE, or are subject to other reactions which may cause decomposition thereof. Accordingly, it is preferred that amphiphile/co-  
15    lipid compositions be stored at low temperature, such as -70 degrees C, until use.

To produce a dispersed suspension, the lipid film was then hydrated with sterile deionized water (1 ml) for 10 minutes, and then vortexed for 1 minute (sonication for 10 to 20 seconds in a bath sonicator may also be  
20    used, and sonication has proved useful for other amphiphiles such as DC-chol). The resulting suspension was then diluted with 4 ml of water to yield a solution that is 670 $\mu$ M in cationic amphiphile and 670 $\mu$ M in neutral colipid.

Experiments were also performed using spermine cholesterol  
25    carbamate (amphiphile No. 67) and other amphiphiles of the invention. With respect to spermine cholesterol carbamate, the optimum molar ratio of amphiphile to DOPE under the conditions tested was determined to be 1:2,

not 1:1. Optimized ratios for many of the amphiphiles of the invention are reported in Figures 13, 14 and 15, and are readily determined by those skilled in the art.

For preparation of the transfecting solution, DNA encoding for  $\beta$ -galactosidase (pCMV $\beta$ , ClonTech., Palo Alto, CA) was dissolved in  
5 OptiMEM culture medium (Gibco/ BRL No. 31885-013). The resulting solution had a DNA concentration of 960  $\mu$ M (assuming an average molecular weight of 330 daltons for nucleotides in the encoding DNA).

The following procedure was used to test a 1:1 molar mixture of the  
10 cationic amphiphile spermidine cholesterol carbamate in combination with DOPE. A 165  $\mu$ l aliquot of spermidine cholesterol carbamate (670  $\mu$ M) containing also the colipid ( at 670  $\mu$ M ) was pipetted into 8 separate wells in a 96-well plate containing OptiMEM (165 $\mu$ l) in each well. The resulting 335  $\mu$ M solutions were then serially diluted 7 times to generate 8 separate  
15 amphiphile-containing solutions having concentrations ranging from 335  $\mu$ M to 2.63  $\mu$ M, with each resultant solution having a volume of 165  $\mu$ l. Thus, 64 solutions were prepared in all, there being 8 wells each of 8 different concentrations of amphiphile/DOPE.

Independently, DNA solutions (165 $\mu$ l, 960 $\mu$ M) were pipetted into 8  
20 wells containing OptiMEM (165  $\mu$ l), and the resulting 480 $\mu$ M solutions were then serially diluted 7 times to generate 8 separate 165  $\mu$ l solutions from each well, with the concentrations of DNA in the wells ranging from 480  $\mu$ M to 3.75  $\mu$ M.

The 64 test solutions (cationic amphiphile: neutral lipid) were then  
25 combined with the 64 DNA solutions to give separate mixtures in 64 wells, each having a volume of 330 $\mu$ l, with DNA concentrations ranging from 240  $\mu$ M to 1.875  $\mu$ M along one axis, and lipid concentrations ranging from 167



$\mu\text{M}$  to  $1.32 \mu\text{M}$  along the other axis. Thus 64 solutions were prepared in all, each having a different amphiphile: DNA ratio and/or concentration. The solutions of DNA and amphiphile were allowed to stand for 15 to 30 minutes in order to allow complex formation.

5           A CFT-1 cell line (human cystic fibrosis bronchial epithelial cells immortalized with papillomavirus) provided by Dr. James Yankaskas, University of North Carolina, Chapel Hill, was used for the *in vitro* assay. The cells are homozygous for a mutant allele (deletion of phenylalanine at position 508, hereinafter  $\Delta$  F508 ) of the gene encoding for cystic fibrosis transmembrane conductance regulator ("CFTR") protein. CFTR is a cAMP-regulated chloride ( $\text{Cl}^-$ ) channel protein. Mutation of the CFTR gene results typically in complete loss ( or at least substantial impairment) of  $\text{Cl}^-$  channel activity across, for example, cell membranes of affected epithelial tissues.

10           The  $\Delta$  F508 mutation is the most common mutation associated with cystic fibrosis disease. For a discussion of the properties of the  $\Delta$  F508 mutation and the genetics of cystic fibrosis disease see, in particular, Cheng et al., Cell, 63, 827-834 (1990). See also Riordan et al., Science, 245, 1066-1073 (1989); published European Patent Application No. 91301819.8 of Gregory et al., bearing publication number 0 446 017 A1; and Gregory et al., Nature, 20   347, 382-385 (1990).

          The cells were cultured in Hams F12 nutrient media (Gibco/ BRL No. 31765-027) supplemented with 2% fetal bovine serum ("FBS", Irvine Scientific, No. 3000) and 7 additional supplements. Cells were then plated into 96-well tissue culture plates at a density of approximately 7,500 cells/well. Before being used in the assay, cells were allowed to grow for periods of 5-7 days until a confluent pattern had been achieved.

25

Following the allotted time period, three 96-well plates with CFT-1 cells were aspirated in order to remove the growth medium. The various concentrations of DNA-lipid complex (in 100  $\mu$ l aliquots) were transferred to each of three 96-well plates bringing the DNA-lipid complexes in contact with the cells. DNA-only/cell and lipid-only/cell control wells were also prepared on one of the three plates.

The 100  $\mu$ l solutions of DNA-lipid complex were maintained over the cells for 6 hours, after which 50  $\mu$ l of 30% FBS (in OptiMEM) was added to each well. After a further 20-hour incubation period, an additional 100  $\mu$ l of 10% FBS in OptiMEM was also added. Following a further 24-hour incubation period, cells were assayed for expression of protein and  $\beta$ -galactosidase.

For the assays, the resultant medium was removed from the plates and the cells washed with phosphate buffered saline. Lysis buffer (50  $\mu$ l, 250 mM Tris-HCl, pH 8.0, 0.15% Triton X-100) was then added, and the cells were lysed for 30 minutes. The 96-well plates were carefully vortexed for 10 seconds to dislodge the cells and cell debris, and 5  $\mu$ l volumes of lysate from each well were transferred to a plate containing 100  $\mu$ l volumes of Coomassie Plus® protein assay reagent (Pierce Company, No. 23236). The protein assay plates were read by a Bio-Rad Model 450 plate-reader containing a 595nm filter, with a protein standard curve included in every assay.

The level of  $\beta$ -galactosidase activity in each well was measured by adding phosphate buffered saline (50  $\mu$ l) to the remaining lysates, followed by addition of a buffered solution consisting of chlorophenol red galactopyranoside (100  $\mu$ l, 1 mg per ml, Calbiochem No. 220588), 60 mM disodium hydrogen phosphate pH 8.0, 1 mM magnesium sulfate, 10 mM potassium chloride, and 50 mM 2-mercaptoethanol. The chlorophenol red

galactopyranoside, following enzymatic ( $\beta$ -galactosidase) hydrolysis, gave a red color which was detected by a plate-reader containing a 570 nm filter. A  $\beta$ -galactosidase (Sigma No. G6512) standard curve was included to calibrate every assay.

- 5        Following subtraction of background readings, optical data determined by the plate-reader allowed determination of  $\beta$ -galactosidase activity and protein content. In comparison to the amount of  $\beta$ -galactosidase expressed by known transfectants, for example, DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide),
- 10       compounds of the invention are particularly effective in transfecting airway epithelial cells and inducing therein  $\beta$ -galactosidase expression. Relative to DMRIE:DOPE (1:1), the spermidine cholesterol carbamate: DOPE mixture (also 1:1) demonstrated transfection efficiency improved by a factor of about 5 (see, for example, Figures 13, 14 and 15).

15       Example 2 - Transfection of the Gene Encoding for Human Cystic Fibrosis Transmembrane Conductance Regulator Protein

- The ability of the cationic amphiphiles of the invention to transfect cells and to induce therein biochemical corrections was demonstrated with a separate *in vitro* assay. Immortalized human cystic fibrosis airway cells
- 20       (CFT-1, as above) were used.

- In preparation for the assay, the cells were grown on glass coverslips until approximately 60% confluent. The cells were then transfected with a complex of spermidine cholesterol carbamate:DOPE (1:1) and a plasmid(pCMV- CFTR) containing a cDNA that encodes wild type human
- 25       CFTR. pCMV-CFTR plasmid is a construct containing the encoding sequence for CFTR and the following regulatory elements, a CMV promoter and enhancer, and an SV40 polyadenylation signal. Additional constructs

suitable for the practice of this example include pMT-CFTR, Cheng et al., Cell, 63, 827-834 (1990). The complex used was 10.5  $\mu$ molar of spermidine cholesterol carbamate (also of DOPE) and 30  $\mu$ molar of pCMV-CFTR based on nucleotide.

5           48 hours after amphiphile-mediated transfection, cells were tested for cAMP-stimulated  $\text{Cl}^-$  channel activity using the 6-methoxy-N-(3-sulfopropyl)quinolinium ("SPQ") assay. See S. Cheng et al., Cell, 66, 1027-1036 (1991) for further information concerning assay methodology. In the assay, cAMP-dependent  $\text{Cl}^-$  channel activity was assessed using "SPQ"

10           (from Molecular Probes, Eugene, Oregon), a halide-sensitive fluorophore. Increases in halide permeability results in a more rapid increase in SPQ fluorescence, and the rate of change (rather than the absolute change in fluorescence) is the important variable in assessing  $\text{Cl}^-$  permeability. See also Rich et al., Nature, 347, 358-363 (1990) for background information.

15           Fluorescence of the SPQ molecule in individual cells was measured using an inverted microscope, Nikon, , a digital imaging system from Universal Imaging, and an ICCD camera, Hamamatsu, Inc.. Cells were selected for analysis without prior knowledge of their expected rate-of-change- in-fluorescence characteristics.

20           In each experiment, up to five microscope fields of between 90 and 100 cells were examined on a given day, and studies under each condition were repeated on at least 3 different days. Since expression of CFTR is heterogenous (i.e. cells do not produce identical amounts of CFTR), the data presented were for the 20% of cells in each field exhibiting the greatest

25           response.

As expected, cells that were mock transfected failed to exhibit any measurable increase in cAMP-stimulated halide fluorescence. In contrast,

cells that had been transfected with the wild type CFTR cDNA displayed a rapid increase in SPQ fluorescence upon stimulation with cAMP agonist, indicating increased permeability to anions. Approximately 60% of the cells assayed exhibited measurable cAMP-stimulated Cl<sup>-</sup> channel activity.

- 5 Accordingly, spermidine cholesterol carbamate, and other cationic amphiphiles of the invention similarly tested, are effective in transferring CFTR-encoding plasmid into immortalized CF airway cells.

### Example 3 - CAT Assay

#### part A

- 10 This assay was used to assess the ability of the cationic amphiphiles of the invention to transfect cells in vivo from live specimens. In the assay, the lungs of balb/c mice were instilled intra-nasally (the procedure can also be performed trans-tracheally) with 100 µl of cationic amphiphile:DNA complex, which was allowed to form during a 15-minute period prior to
- 15 administration according to the following procedure. The amphiphile (premixed with co-lipid, see below) was hydrated in water for 10 minutes, a period sufficient to yield a suspension at twice the final concentration required. This was vortexed for two minutes and aliquoted to provide 55 microliter quantities for each mouse to be instilled. Similarly, DNA
- 20 encoding the reporter (CAT) gene was diluted with water to a concentration twice the required final concentration, and then aliquoted at 55 microliters for each mouse to be instilled. The lipid was gently combined with the DNA (in a polystyrene tube), and the complex allowed to form for 15 minutes before the mice were instilled therewith.
- 25 The plasmid used (pCMVHI-CAT, see Example 4) provides an encoding DNA for chloramphenicol transferase enzyme. Specifics on the amphiphile:DNA complexes are provided below.

Two days following transfection, mice were sacrificed, and the lungs and trachea removed, weighed, and homogenized in a buffer solution (250 mM Tris, pH 7.8, 5mM EDTA). The homogenate was clarified by centrifugation, and the deacetylases therein were inactivated by heat treatment at 70 °C for 10 minutes. Lysate was incubated overnight with acetyl coenzyme A and C<sup>14</sup> –chloramphenicol. CAT enzyme activity was then visualized by thin layer chromatography ("TLC") following an ethyl acetate extraction. Enzyme activity was quantitated by comparison with a CAT standard curve.

10        The presence of the enzyme CAT will cause an acetyl group to be transferred from acetylcoenzyme A to C<sup>14</sup> –chloramphenicol. The acetylated/radiolabeled chloramphenicol migrates faster on a TLC plate and thus its presence can be detected. The amount of CAT that had been necessary to generate the determined amount of acetylated chloramphenicol  
15        can then be calculated from standards.

      The activity of spermidine cholesterol carbamate (amphiphile No.53) was determined in the CAT assay in relation to the recognized transfection reagents DMRIE and DC-Chol. Figure 10 demonstrates dramatically (as ng CAT activity per 100 mg tissue) the enhanced ability of spermidine  
20        cholesterol carbamate (amphiphile No. 53) to transfect cells in vivo, which enhancement is about 20-fold, or greater, in this assay. In the assay, activity was measured as ng CAT enzyme per 100 mg lung tissue. As a comparison, it is generally observed that DMRIE, a well known transfectant, when prepared as a 1:1 molar mixture with DOPE and then complexed with  
25        plasmid DNA (1.7 mM DMRIE, 1.7 mM DOPE, 1.2 mM plasmid DNA measured as nucleotide) gives about 1 to 2 ng activity per 100 mg lung tissue in this assay.

With respect to the comparison provided by Figure 10, the following conditions are of note. The transfection solution for spermidine cholesterol carbamate contained 6mM DNA measured as concentration of nucleotide, and 1.5 mM of cationic amphiphile. Following generally the procedure of

5 Example 1, each amphiphile had also been premixed with DOPE, in this case at 1:1 molar ratio. For transfection with DC-chol, the molar ratio of DC-chol to DOPE was 3:2, and the concentrations of cationic amphiphile and of DNA (as nucleotide) were 1.3 mM and 0.9 mM, respectively. For

10 transfection with DMRIE, the molar ratio of DMRIE to DOPE was 1:1 and the concentrations of cationic amphiphile and of DNA were 1.7 mM and 1.2 mM, respectively. These concentrations (and concentration ratios) for each amphiphile, and colipid and DNA, had been determined to be optimal for transfection for that respective amphiphile, and accordingly were used as the basis for the comparison presented herein.

15 For spermidine cholesterol carbamate (amphiphile No. 53), optimization experiments were also performed to determine preferred concentrations of plasmid for a particular amphiphile concentration (see Figure 11), and also to determine preferred concentrations of the same amphiphile in relation to a particular plasmid concentration (see Figure 12).

20 Transfection efficiency was optimal at an amphiphile concentration of 1.5 mM (DOPE also being present at 1.5 mM), and about 6 mM (by nucleotide) of plasmid, or about at a ratio of 1:4. It was noted, however, that concentrations of about 0.75 mM of amphiphile, and 3.0 mM of plasmid were less toxic to the target cells.

25 Intra-nasal transfection with pCMVHI-CAT vector was also performed in mice using spermidine cholesterol carbamate as cationic amphiphile but with cholesterol as co-lipid. In this experiment, the

concentrations of spermidine cholesterol carbamate tested were between 1.0 and 1.5 mM (cholesterol being present at a 1:1 molar ratio in each case, with the mixing of amphiphile and co-lipid being performed as above). The DNA concentration (measured as nucleotide concentration) was between 4.0 and 6.0 mM. Transfection efficiency (again measured as ng CAT/100 mg tissue) was less effective than with DOPE as co-lipid; however, the transfections were substantially more effective than those achieved using DC-Chol/DOPE.

#### part B

Additional experiments were performed to compare in vivo the transfection efficiency of cationic amphiphiles depicted in Figures 1, 5 and 7. Results therefor are reported in Figures 13, 14 and 15 respectively. The compounds were administered intra-nasally using between 12 and 15 mice per compound. As in part A above, ng CAT activity was measured per 100 mg of tissue. However, improved vectors (pCF1/CAT and its near equivalent pCF2/CAT) were used. In part resulting from improved vector performance, incubations of lysate with acetyl coenzyme A and C<sup>14</sup> chloramphenicol were conducted for only 30 minutes. Construction of pCF1/CAT and pCF2/CAT is described below in Example 4.

The in vivo data reported in Figures 13, 14 and 15 were compiled generally as follows. As aforementioned, Figures 10 and 11 report data from the complete in vivo optimization of amphiphile No. 53. Amphiphile No. 67 was subjected to a similar partial optimization. With respect to all of the other cationic amphiphiles reported on, and taking advantage of numerous structural similarities, optimized compositions for in vivo testing were extrapolated from in vitro results. This facilitated the screening of large numbers of amphiphiles and produced broadly, if not precisely,



- comparable data. For all amphiphiles other than Nos. 53 and 67, the ratio, for in vivo testing, of amphiphile concentration to DOPE concentration was taken from the in vitro experiments, as was the optimized ratio of amphiphile concentration to DNA concentration (see Example 1).
- 5 Accordingly, for such amphiphiles the in vivo test concentration was fixed at 1mM, thereby fixing also the co-lipid concentration. [Broadly, the molar ratio of the amphiphile to co-lipid DOPE ranged from 1:2 (for example, spermine cholesterol carbamate, No. 67) through 1:1 (for example, spermidine cholesterol carbamate, No. 53) to about 2:1 (for example,
- 10 amphiphile No. 75)]. The concentration of plasmid DNA varied for each amphiphile species tested in order to duplicate the optimized amphiphile/DNA ratio that had been determined in vitro.

part C

- That the novel amphiphiles of the invention are an important
- 15 contribution to the art is immediately seen by comparing their performance - as in vivo transfection enhancers - to that of closely related cationic amphiphiles that lack the novel T-shape. It has been determined that spermidine cholesterol carbamate provides a much greater level of enhancement than N<sup>1</sup>-spermidine cholesteryl carbamate which contains the
- 20 same number of carbon and nitrogen atoms in its cationic alkylamine component but which is linear and not "T-shaped". Following generally the procedures of Example 3, part B, and using respectively 6mM (as nucleotide), 1.5 mM, and 1.5 mM concentrations of DNA, amphiphile and of co-lipid, the transfection enhancement provided by spermidine cholesterol
- 25 carbamate (amphiphile No.53) , in relation to N<sup>1</sup>-spermidine cholesteryl carbamate, was determined to be about 30 fold.

Also following the procedures of Example 3, part B, and using respectively 4mM (as nucleotide), 1mM, and 2 mM concentrations of DNA, amphiphile and co-lipid, the transfection enhancement provided by spermine cholesterol carbamate (amphiphile No. 67) — in relation to N<sup>1</sup>-thermospermine cholesteryl carbamate and N<sup>1</sup>-spermine cholesteryl carbamate to which spermine cholesterol carbamate is similarly related — is at least about 30 fold.

#### Example 4- Construction of vectors

As aforementioned, numerous types of biologically active molecules can be transported into cells in therapeutic compositions that comprise one or more of the cationic amphiphiles of the invention. In an important embodiment of the invention, the biologically active macromolecule is an encoding DNA. There follows a description of novel vectors (plasmids) that are preferred in order to facilitate expression of such encoding DNAs in target cells.

##### part A — pCMVHI-CAT

pCMVHI-CAT is representative of plasmid constructs useful in the practice of the invention. Although the plasmid is provided in a form carrying a reporter gene (see Example 3), transgenes having therapeutic utility may also be included therein.

The pCMVHI-CAT vector is based on the commercially available vector pCMV $\beta$  (Clontech). The pCMV $\beta$  construct has a pUC19 backbone (J. Vieira, et al., Gene, 19, 259-268, 1982) that includes a procaryotic origin of replication derived originally from pBR322. Basic features of the pCMVHI-CAT plasmid (as constructed to include a nucleotide sequence coding for CAT) are as follows. Proceeding clockwise — the human cytomegalovirus immediate early gene promoter and enhancer, a fused tripartite leader from

adenovirus and a hybrid intron, a linker sequence, the CAT cDNA, an additional linker sequence, the late SV40 polyadenylation signal, and the pUC origin of replication and backbone that includes the gene for ampicillin resistance.

5           The human cytomegalovirus immediate early gene promoter and enhancer spans the region from nucleotides 1-639. This corresponds to the region from -522 to +72 relative to the transcriptional start site (+1) and includes almost the entire enhancer region from -524 to -118 as originally defined by Boshart et al, Cell 41:521-530, 1985. The CAAT box is located at  
10   nucleotides 487-491 and the TATA box is at nucleotides 522-526 in pCMVHI-CAT. The CAT transcript is predicted to initiate at nucleotide 549, which is the transcriptional start site of the CMV promoter. The tripartite leader-hybrid intron is composed of a fused tri-partite leader from adenovirus containing a 5' splice donor signal, and a 3' splice acceptor signal  
15   derived from an IgG gene. The elements in the intron are as follows: the first leader, the second leader, part of the third leader, the splice donor sequence and intron region from the first leader, and the mouse immunoglobulin gene splice donor sequence. The length of the intron is 230 nucleotides. The CAT coding region comprises nucleotides 1257-1913. The  
20   SV40 poly A signal extends from nucleotide 2020 to 2249.

Accordingly, construction of the pCMVHI-CAT plasmid proceeded as follows. The vector pCMV $\beta$  (Clontech, Palo Alto, CA) was digested with Not I to excise the  $\beta$ -galactosidase gene. The vector fragment lacking the  $\beta$ -galactosidase gene was isolated and ligated to form pCMV.

25           The hybrid intron (Figure 17) was obtained from the plasmid pAD $\beta$  (Clontech). The hybrid intron had been isolated from a 695 base pair XhoI-EcoRI fragment of p91023(B), see Wong et al., *Science*, 228, 810-815 (1985).

The hybrid intron contains the fused tripartite leader from adenovirus, the donor site from the first segment of the tripartite leader, and the acceptor site from an IgG gene, and has a length of 230 bp.

pAD $\beta$  was digested with Pml I and Not I, and the ~500 base-pair (bp) fragment was isolated, and then ligated into the Not I site of pBluescriptII KS(-) (Stratagene, La Jolla, CA) to form pBlueII-HI.

pBlueII-HI was digested with XhoI and NotI to excise the hybrid intron fragment. This fragment was ligated into the XhoI and NotI sites of pCMV, replacing the SV40 intron to form pCMVHI.

The CAT gene was obtained from the Chloramphenicol Acetyltransferase GenBlock (Pharmacia, Piscataway, NJ). This 792 bp Hind III fragment was blunted with the Klenow fragment of DNA Polymerase I, then Not I linkers (New England Biolabs) were ligated to each end. After digestion with Not I to expose the Not I sticky ends, the fragment was subcloned into the Not I site of pCMV to form pCMV-CAT. pCMV-CAT was digested with Not I to excise the CAT fragment. The CAT fragment was ligated into pCMVHI to form pCMVHI-CAT which is depicted in Figure 16.

part B — pCF1 and pCF2

Although pCMVHI is suitable for therapeutic transfections, further performance enhancements (including increased expression of transgenes) are provided by the pCF1 and pCF2 plasmids. A map of pCF1/CAT is shown in Figure 18, panel A, and a map of pCF2/CAT is shown in panel B.

Briefly, pCF1 contains the enhancer/promoter region from the immediate early gene of cytomegalovirus (CMV). A hybrid intron is located between the promoter and the transgene cDNA. The polyadenylation signal of the bovine growth hormone gene was selected for

placement downstream from the transgene. The vector also contains a drug-resistance marker that encodes the aminoglycosidase 3'-phosphotransferase gene (derived from the transposon Tn903, A. Oka et al., Journal of Molecular Biology , 147, 217-226, 1981) thereby conferring resistance to kanamycin. Further details of pCF1 structure are provided directly below, including description of placement therein of a cDNA sequence encoding for cystic fibrosis transmembrane conductance regulator (CFTR) protein.

The pCF1 vector is based on the commercially available vector pCMV $\beta$  (Clontech). The pCMV $\beta$  construct has a pUC19 backbone (J. Vieira, et al., Gene , 19, 259-268, 1982) that includes a procaryotic origin of replication derived originally from pBR322.

Basic features of the pCF1-plasmid (as constructed to include a nucleotide sequence coding for CFTR) are as follows. Proceeding clockwise — the human cytomegalovirus immediate early gene promoter and enhancer, a fused tripartite leader from adenovirus and a hybrid intron, a linker sequence, the CFTR cDNA, an additional linker sequence, the bovine growth hormone polyadenylation signal, pUC origin of replication and backbone, and the kanamycin resistance gene. The pCF1-CFTR plasmid has been completely sequenced on both strands.

The human cytomegalovirus immediate early gene promoter and enhancer spans the region from nucleotides 1-639. This corresponds to the region from -522 to +72 relative to the transcriptional start site (+1) and includes almost the entire enhancer region from -524 to -118 as originally defined by Boshart et al., Cell 41, 521-530 (1985). The CAAT box is located at nucleotides 486-490 and the TATA box is at nucleotides 521-525 in pCF1-

CFTR. The CFTR transcript is predicted to initiate at nucleotide 548, which is the transcriptional start site of the CMV promoter.

The hybrid intron is composed of a fused tri-partite leader from adenovirus containing a 5' splice donor signal, and a 3' splice acceptor signal  
5 derived from an IgG gene. The elements in the intron are as follows: the first leader (nucleotides 705-745), the second leader (nucleotides 746-816), the third leader (partial, nucleotides 817-877), the splice donor sequence and intron region from the first leader (nucleotides 878-1042), and the mouse immunoglobulin gene splice donor sequence (nucleotides 1043-1138). The  
10 donor site (G|GT) is at nucleotides 887-888, the acceptor site (AG|G) is at nucleotides 1128-1129, and the length of the intron is 230 nucleotides. The CFTR coding region comprises nucleotides 1183-5622.

Within the CFTR-encoding cDNA of pCF1-CFTR, there are two differences from the originally-published predicted cDNA sequence (J.  
15 Riordan et al., Science, 245, 1066-1073, 1989); (1) an A to C change at position 1990 of the CFTR cDNA which corrects an error in the original published sequence, and (2) a T to C change introduced at position 936. The change at position 936 was introduced by site-directed mutagenesis and is silent but greatly increases the stability of the cDNA when propagated in  
20 bacterial plasmids (R. J. Gregory et al. et al., Nature, 347, 382-386, 1990). The 3' untranslated region of the predicted CFTR transcript comprises 51 nucleotides of the 3' untranslated region of the CFTR cDNA, 21 nucleotides of linker sequence and 114 nucleotides of the BGH poly A signal.

The BGH poly A signal contains 90 nucleotides of flanking sequence  
25 5' to the conserved AAUAAA and 129 nucleotides of flanking sequence 3' to the AAUAAA motif. The primary CFTR transcript is predicted to be cleaved downstream of the BGH polyadenylation signal at nucleotide 5808.

There is a deletion in pCF1-CFTR at position +46 relative to the cleavage site, but the deletion is not predicted to effect either polyadenylation efficiency or cleavage site accuracy, based on the studies of E.C. Goodwin et al., J. Biol. Chem., 267, 16330-16334 (1992). After the addition of a poly A  
5 tail, the size of the resulting transcript is approximately 5.1 kb.

pCF2 plasmid, Figure 18 (B), contains a second CMV enhancer, in tandem with the first. Enhanced expression of transgenes from pCF1 or pCF2 results from the combination of a strong promoter, the presence of a highly efficient polyadenylation signal, a leader sequence that enhances  
10 translation, and an intron to increase message stability.

Example 5- Correction of Chloride Ion Transport Defect in Nasal Polyp Epithelial Cells of a Cystic Fibrosis Patient by Cationic Amphiphile-Mediated Gene Transfer

Primary (non-immortalized) nasal polyp cells from an adult male  
15 cystic fibrosis patient (homozygous for the  $\Delta$  F508 mutation) were grown on collagen-coated permeable filter supports (Millicells) to form a polarized and confluent epithelial monolayer. Once the monolayer was electrically tight (about 5 to 7 days post seeding, and as indicated by the development of resistance across the cell sheet), the apical surface can be exposed to  
20 formulations of cationic amphiphile: DNA complex.

In this case, the amphiphile (spermidine cholesterol carbamate ) was provided as a 1:1 (by mole) mixture with DOPE, and this mixture was then complexed with pCMV-CFTR plasmid vector (a construct encoding wild type human cystic fibrosis transmembrane conductance regulator protein,  
25 see above). Concentrations in the final mixture were 42  $\mu$ molar of spermidine cholesterol carbamate (and also of DOPE) and 60  $\mu$ molar (based on molarity in nucleotides) of the plasmid expression vector.

Expression of CFTR was determined by measuring cAMP-stimulated transepithelial chloride secretion in a modified Ussing chamber, Zabner et al., Nature Genetics ,6, 75-83 (1984). The mucosal side of the epithelium was bathed in Ringer's bicarbonate solution bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

- 5 The composition of the submucosal solution was similar to the mucosal solution with the exception that sodium gluconate replaced sodium chloride. Transepithelial voltage was clamped to 0 mV and short circuit current was recorded. Amiloride (10  $\mu$ M) was applied into the apical bath, followed by the mucosal addition of forskolin and IBMX (at 100  $\mu$ M each).
- 10 5-nitro-2-(3-phenylpropylamino) benzoic acid ("NPPB") , an inhibitor of CFTR chloride channels, was then added to the mucosal solution at 10 to 30  $\mu$ M.

Chloride secretion (i.e. movement of chloride from the epithelial cells to the mucosal solution) is shown as an upward deflection (see Figure 19).

- 15 The same plasmid vector, but containing a reporter gene, was used as a negative control. A cAMP-stimulated current (0.5 to 2.5  $\mu$ ampere/cm<sup>2</sup>) was observed in monolayers transfected with wild type CFTR gene. Current was not detected with the pCMV- $\beta$ -galactosidase control.

Example 6- Correction of Chloride Ion Transport Defect in Airway  
20 Epithelial Cells of a Cystic Fibrosis Patient by Cationic Amphiphile-  
Mediated Gene Transfer

A recommended procedure for formulating and using the pharmaceutical compositions of the invention to treat cystic fibrosis in human patients is as follows.

- 25 Following generally the procedures described in Example 1, a thin film (evaporated from chloroform) is produced wherein spermine cholesterol carbamate (amphiphile No. 67) and DOPE are present in the



molar ratio of 1: 2. The amphiphile-containing film is rehydrated in water-for-injection with gentle vortexing to a resultant amphiphile concentration of about 3mM. However, in order to increase the amount of amphiphile/DNA complex that may be stably delivered by aerosol as a homogeneous phase (for example, using a Puritan Bennett Raindrop nebulizer from Lenexa Medical Division, Lenexa, KS, or the PARI LC Jet™ nebulizer from PARI Respiratory Equipment, Inc., Richmond, VA), it may be advantageous to prepare the amphiphile-containing film to include also one or more further ingredients that act to stabilize the final amphiphile/DNA composition. Accordingly, it is presently preferred to prepare the amphiphile-containing film as a 1: 2 : 0.05 molar mixture of amphiphile No. 67, DOPE, and PEG(5000)-DMPE. [A suitable source of PEG-DMPE, polyethylene glycol 5000 - dimyristoylphosphatidyl ethanolamine, is Catalog No. 880210 from Avanti Polar Lipids, Alabaster, AL]. Additional fatty acid species of PEG-PE may be used in replacement therefor.

Without being limited as to theory, PEG(5000)-DMPE is believed to stabilize the therapeutic compositions by preventing further aggregation of formed amphiphile/DNA complexes. Additionally it is noted that PEG(2000)-DMPE was found to be less effective in the practice of the invention.

pCF1-CFTR plasmid (containing an encoding sequence for human cystic fibrosis transmembrane conductance regulator, see Example 4) is provided in water-for-injection at a concentration, measured as nucleotide, of 4 mM. Complexing of the plasmid and amphiphile is then allowed to proceed by gentle contacting of the two solutions for a period of 10 minutes.

It is presently preferred to deliver aerosolized DNA to the lung at a concentration thereof of between about 2 and about 12 mM (as nucleotide). A sample of about 10 to about 40 ml is generally sufficient for one aerosol administration to the lung of an adult patient who is homozygous for the  $\Delta$  F508 mutation in the CFTR-encoding gene.

It is expected that this procedure (using a freshly prepared sample of amphiphile/DNA) will need to be repeated at time intervals of about two weeks, but depending considerably upon the response of the patient, duration of expression from the transfected DNA, and the appearance of any potential adverse effects such as inflammation, all of which can be determined for each individual patient and taken into account by the patient's physicians.

One important advantage of the cationic amphiphiles of the present invention is that they are substantially more effective — in vivo — than other presently available amphiphiles, and thus may be used at substantially lower concentrations than known cationic amphiphiles. There results the opportunity to substantially minimize side effects (such as amphiphile toxicity, inflammatory response) that would otherwise affect adversely the success of the gene therapy. A further particular advantage associated with use of many of the amphiphiles of the invention should again be mentioned. Many of the amphiphiles of the invention were designed so that the metabolism thereof would rapidly proceed toward relatively harmless biologically-compatible components. In this regard, highly active amphiphiles 53, 67, and 75 are of particular note.

Example 7- Further Enhancements in Plasmid Design for Gene Therapy :

Replicating Episomal Plasmids

Although the above design features substantially enhance the performance of available plasmids, further modifications are desirable in order that therapeutic compositions comprising such plasmids and cationic amphiphiles have optimal performance for gene therapy.

It is desirable that plasmids for gene therapy also be able to replicate in the cells of patients, since continued presence of the plasmid will provide correction of the genetic defect (in the case of cystic fibrosis, lack of functioning CFTR protein in the cell membrane of lung epithelial cells or other cells) over an extended period of time. There is concern that plasmids representative of the current art (that is, those that cannot replicate in the targeted cells of a patient) may be degraded after only a relatively short period of maintenance in the patient, thus requiring excessive repeat administrations.

Long term correction could perhaps be achieved using a vector designed to integrate into chromosomes in the patient's targeted cells (for example, vectors patterned on retrovirus). Such a strategy, however, involves risks including (1) that the vector will integrate into an essential region of a chromosome, or (2) that the vector will integrate adjacent to an oncogene and activate it.

Accordingly, it would be desirable to provide for continued maintenance of gene therapy vectors (plasmids) in target cells by other means. One such strategy is to construct a plasmid capable of being maintained separately in the nucleus of a target cell, and that is also able to replicate there (i.e. an episome).

Plasmids provided according to this aspect of the invention can be constructed as follows. It has been determined (C. McWhinney et al., Nucleic Acids Research, 18, 1233-1242, 1990) that the 2.4 kb HindIII-XhoI fragment that is present immediately 5' to exon 1 of the human c-myc gene contains an origin of replication. The fragment was then cloned into a plasmid that if transfected into HeLa cells was shown to persist therein for more than 300 generations under drug selection. Replication was shown to be semiconservative (C. McWhinney et al.). Although approximately 5% of the plasmid population was lost per cell generation without drug selection in those experiments, this result nonetheless demonstrates substantial stabilization would be of benefit with respect to the design of therapeutic plasmids for gene therapy.

Accordingly, in one example of a replicating episomal vector, a variant of pCF1-CFTR (or pCF1-CAT) can be constructed in which a copy of the 2.4 kb HindIII-XhoI fragment is placed just 5' to the CMV enhancer/promoter region of the pCF1 backbone. Alternatively, between 2 and about 4 - in tandem - copies of the 2.4 kb fragment may be similarly positioned. The increase in plasmid size that results from insertion of the 2.4 kb fragment (or multiple copies thereof) is predicted to provide an additional benefit, that is, to facilitate plasmid unwinding, thus facilitating the activity of DNA polymerase.

Use of this origin of replication, or multiple copies thereof, allows the resultant plasmid to replicate efficiently in human cells. Other DNA sequences containing other origins of replication may also be used (for example, as found in the human  $\beta$ -globin gene, or the mouse DHFR gene.

A plasmid that can be constructed according to this aspect of the invention and containing the cytomegalovirus promoter and enhancer, an

intron, the CFTR cDNA, the bovine growth hormone polyadenylation signal, the kanamycin resistance transposon Tn903, and 4 copies of the 2.4 kb 5' flanking region of the human c-myc gene is shown in Figure 20.

Example 8- Further Enhancements in Plasmid Design for Gene Therapy :

5

Use of Cytokine Promoters to Modulate Expression  
of Transgenes in Gene Therapy

Chronic inflammation is associated with numerous of the disease  
10 states that can be treated by gene therapy. Representative of such disease  
states are cystic fibrosis (using CFTR), bronchitis, adult respiratory distress  
syndrome (using alpha-1 antitrypsin), and metastatic cancers (through  
upregulation of p53, TIMP-1, and TIMP-2). Inflammatory conditions  
typically involve many interrelated processes (for example, involvement by  
15 many types of immune system cells and liver proteins), whereby the body  
attempts to heal a damaged or infected tissue. However, chronic  
inflammation which persists as a result of an unresolved condition may lead  
to permanent tissue damage, as is the case with respect to lung tissue  
affected by cystic fibrosis and associated and unresolved lung infections. In  
20 fact, permanent damage to the lung tissue of cystic fibrosis patients is a  
leading cause of their mortality. It would be desirable to provide gene  
therapy in such a manner as to treat inflammatory conditions associated  
with the targeted disease state.

Accordingly, a further aspect of the present invention involves  
25 construction of gene therapy vectors in which the therapeutic transgene is  
placed under control of an RNA polymerase promoter from a cytokine gene  
(or a gene that encodes another similar regulatory protein) such as, for  
example, the promoter for any of interleukin 2, interleukin 8, interleukin 1,

interleukin 11, interleukin 6, endothelin -1, monocyte chemoattractant protein -1, IL-1ra (receptor agonist), or for GM-CSF.

Cytokines may be defined as hormone-like intercellular signal proteins that are involved in regulation of cell proliferation, differentiation, and function, such as concerning haematopoiesis and lymphopoiesis. The interleukins are a particular group of cytokines having promoters that are useful in the practice of the invention. The interleukins are proteins, typically of unrelated origin, which act as intercellular signals mediating reactions between immunoreactive cells. However, it is understood that many "interleukins" have effects upon additional cell types including endothelial cells, epithelial cells, and fibroblasts.

Since the concentration of many cytokines is upregulated at an affected site in response to the level of inflammation that is present, gene therapy vectors can be designed wherein the level of therapeutic transgene expressed therefrom is determined, in part, by the level of inflammation present. There follows hereafter description of how such vectors are designed using primarily properties of the interleukin 8 gene as an example.

It has been determined that numerous biologically active molecules are present in tissues at concentrations thereof that increase with the severity of an inflammatory condition (for example, tumor necrosis factor "TNF" and potentially transcription factors such as NF-kB, AP-1, NF-IL6 and octamer binding protein).

It has also been determined that interleukin 8, a polypeptide of 8,500 MW, is upregulated by inflammation and acts as a potent chemoattractant for T lymphocytes and neutrophil cells that are themselves involved in the inflammation response. The interleukin 8 gene is regulated primarily at the transcriptional level, and it has also been determined (H. Nakamura et al.,

Journal of Biological Chemistry , 266, 19611-19617, 1991) that TNF can increase interleukin 8 transcription by more than 30 -fold in vitro in bronchial epithelial cells. Accordingly, there follows description of gene therapy vectors which take advantage of the above.

5           A plasmid can be constructed that is substantially similar to pCF1, that is, derived from a pUC plasmid containing a bacterial-derived origin of replication and a gene conferring resistance to kanamycin. The resultant plasmid contains also, in sequence, a CMV enhancer, a promoter, a hybrid intron, a cDNA sequence encoding CFTR, and the bovine growth hormone  
10 polyadenylation signal. As RNA polymerase promoter there is selected the -335 to +54 region of the interleukin 8 promoter. This region gave the highest ratio in terms of promoter activity plus TNF over minus TNF (Nakamura, 1991)

          Such a plasmid has particularly valuable performance attributes. As  
15 inflammation increases in a cystic fibrosis-affected lung (and therefore the need to treat the lung with gene therapy also increases), the concentration of various inflammation-related molecules ( such as TNF) will increase. By placing the CFTR-encoding cDNA of the therapeutic plasmid under the control of a transcriptional promoter (that of interleukin 8, for example) that  
20 is itself sensitive to the concentration of inflammation-related substances in contact with the cell, the promoter will function as a natural gene switch such that the amount of beneficial CFTR transcription will be tailored to the amount of inflammation. As aforementioned, RNA polymerase promoter sequences derived from the other aforementioned genes are also useful in  
25 the practice of the invention.

### Example 9 Intravenous Delivery of Transgenes

For some disease states, such as cystic fibrosis, it is desirable to deliver transgenes to the lung. Delivery by aerosol is the most direct approach to achieve this goal. However, given the difficulties inherent with the delivery of an aerosol together with the potential need to target organs other than the lung (for example, the pancreas for cystic fibrosis), it is important to evaluate the feasibility of lung delivery using non-aerosol delivery formats. Accordingly, intravenous delivery of a reporter transgene was performed using a mouse model and the feasibility of intravenous organ targeting was assessed. A comparison was made of feasibility of delivery to the lung and the heart.

The reporter plasmid pCF-1 CAT (Example 4) was used and was purified to minimize endotoxin ( $<1$  EU/mg pDNA), and also chromosomal DNA contamination ( $<2\%$ ). Amphiphile No. 53 (1:1 with DOPE) / DNA complex was prepared according to the procedures of Example 3. The amphiphile was provided as the free base, the plasmid was prepared as a sodium salt in water, and the DOPE was provided in zwitterionic form.

The animal model was the BALB/c mouse. Females 6-8 weeks old weighing 16-18 g were injected intravenously using the tail vein, using 5 animals per group. The volume of lipid:pDNA complex used was 100  $\mu$ l in all experiments. Unless noted otherwise, mice were sacrificed 48 h following administration of the complex. Organs were frozen immediately on dry ice to store for subsequent analysis.

Expression of chloramphenicol acetyl transferase (CAT) was quantitated using a radiochemical assay for CAT enzymatic activity.



Organs were weighed and homogenized on ice in a lysis buffer containing protease inhibitors. The lysate was freeze-thawed 3X, centrifuged, and heated to 65°C to inactivate deacetylases before adding it to a reaction mixture containing <sup>14</sup>C-chloramphenicol. After  
5 an incubation at 37°C, the mixture was extracted with ethyl acetate, concentrated, spotted onto TLC plates and eluted with CHCl<sub>3</sub>/MeOH. Spots corresponding to the acylated reaction products were quantitated (Betagen) and converted to ng CAT activity using authentic CAT standards.

10 It was surprisingly determined that targeting to the heart could be substantially improved by altering the molar ratio (at a constant DNA concentration of 0.9 mM, measured as nucleotide) of amphiphile/DNA in the therapeutic composition. This information is of value in connection with gene therapy for the heart, such as for  
15 coronary disease. However, targeting to the lung remained relatively constant over a range of amphiphile/DNA ratios, all at constant DNA concentration (Figure 21).

At molar ratios of less than about 0.5, the organ distribution was found to be strongly weighted toward the lung. At this molar ratio, the  
20 zeta potential of the complex is negative (about -30 mV) due, in part, to excess negative charge from the DNA relative to the amphiphile. At an amphiphile/DNA ratio of 1.25, however, where the complex has a positive zeta potential (about +30mV), organ distribution was remarkably altered and substantial expression was found in the heart  
25 (Figure 21).

Zeta potentials of the samples can be measured (using typically 5 measurements per sample) employing a Malvern Zetasizer 4

(Malvern Instruments, Southborough, MA.) and a zeta cell (AZ-104 cell, Malvern Instruments Co.). Dried lipid films containing the cationic lipid and DOPE are hydrated in distilled water (dH<sub>2</sub>O). DNA typically should be diluted to a concentration of about 300  $\mu$ M in  
5 dH<sub>2</sub>O. The DNA solution (1.5 mL) can then be added to an equal volume of cationic lipid vesicles and incubated at room temperature for 10 min. Enough NaCl (for example, 4 mM stock) may be added to result in a final concentration of 1 mM NaCl. If necessary, the sample can be diluted further with 1 mM NaCl (to maintain a photomultiplier  
10 signal below 4000 counts per second), and distilled water can be used in place of the NaCl solutions.

According to this aspect of the invention, amphiphiles No. 53 and No. 67 are among those preferred for use in intravenous targeting of the heart, as are many other amphiphiles selected from Groups I and  
15 II.

#### Example 10- Additional Experimental Procedures

##### (A) Additional synthesis procedure for N<sup>4</sup>-spermine cholesteryl carbamate, amphiphile No. 67

(Synthesis of N<sup>1</sup>,N<sup>12</sup>-diCbz-spermine di-HCl salt)

20 Benzylchloroformate (15 mL, 105 mmol) was dissolved in methylene chloride (335 mL) and placed in a three neck flask under a nitrogen atmosphere. Imidazole (14 g, 206 mmol) was dissolved in methylene chloride (200 mL). The three neck flask was cooled to 0-2 °C using an ice-water bath and the imidazole solution was added gradually over 30 min.  
25 The cooling bath was removed and the mixture stirred at room temperature for 1 hour. Methylene chloride (250 mL) and aqueous citric acid (10%, 250 mL) were added to the mixture. The layers were separated and the organic

layer was washed with aqueous citric acid (10%, 250 mL). The organic fraction was dried over magnesium sulfate and concentrated *in vacuo*. The resulting oil was vacuum dried for 2 hours at ambient temperature. To the oil was added dimethylaminopyridine (530 mg, 4.3 mmol) and methylene chloride (250 mL). The mixture was cooled to 0-2 °C and kept under a nitrogen atmosphere. A solution of spermine (10g, 49 mmol) in methylene chloride (250 mL) was added gradually over 15 minutes, maintaining a reaction temperature of 0-2 °C. The reaction mixture was stirred overnight at ambient temperature and then concentrated *in vacuo*. To the resulting material was added 1M hydrochloric acid (67 mL) and methanol (400 mL). The solution was cooled overnight at 4 °C yielding a white precipitate. The precipitate was isolated by vacuum filtration using Whatman #1 filter paper. The N<sup>1</sup>,N<sup>12</sup>-diCbz-spermine di HCl salt (13.38g, 24.7 mmol, 50% yield) thus obtained was dried under vacuum at ambient temperature for 17 hours.

(Synthesis of N<sup>1</sup>,N<sup>12</sup>-diCbz-N<sup>4</sup>-spermine cholesteryl carbamate)

N<sup>1</sup>,N<sup>12</sup>-diCbz-spermine di HCl salt (13.38g, 24.7 mmol) was dissolved in a chloroform, methanol and water mixture in the ratio 65:25:4 (940 mL). The solution was stirred at room temperature and cholesteryl chloroformate (11g, 24.5 mmol) was added. The solution was stirred at ambient temperature for 1.5 hours and then diluted with 1M sodium hydroxide solution (165 mL). The organic and aqueous layers were separated and the organic layer containing the product was washed with water (110 mL). The organic fraction was dried over sodium sulfate, concentrated *in vacuo* and vacuum dried. The crude oil was purified by chromatography using silica gel (60Å, 1 Kg) . The silica was packed in 10% MeOH / CHCl<sub>3</sub> and the column was eluted with 25% MeOH / CHCl<sub>3</sub>.

Fractions of 900 mL were collected and analyzed by thin layer chromatography. Fractions containing the product ( $R_f = 0.5$  in 20% MeOH / CHCl<sub>3</sub>) were combined and concentrated *in vacuo*. The resulting oil was dried under vacuum for 17 hours to give 8.5g (9.67 mmol, 39% yield) of

5 product.

(Synthesis of N<sup>4</sup>-spermine cholesteryl carbamate)

N<sup>1</sup>,N<sup>12</sup>-diCbz-N<sup>4</sup>-spermine cholesteryl carbamate (8.5g, 9.67 mmol) was dissolved in 200 mL of acetic acid and 1.66 g of 10% Pd on carbon was added. The solution was purged with nitrogen and stirred under hydrogen at atmospheric pressure. The hydrogen was supplied to the reaction flask using a balloon filled with hydrogen gas. The hydrogenolysis was allowed to proceed for 3 hours. The reaction mixture was filtered through Whatman #1 filter paper and the catalyst was washed with 250 mL of 10% acetic acid in ethyl acetate. The filtrate was concentrated *in vacuo* to give a residue, coevaporation with chloroform aids removal of the acetic acid. To the crude product was added 1M sodium hydroxide solution (400 mL) and the solution was extracted three times with 10% MeOH / CHCl<sub>3</sub> (700 mL). The combined organic fractions were washed with water (600 mL) and dried over sodium sulfate. The solution was filtered, concentrated *in vacuo* and vacuum dried at ambient temperature for 48 hours. The crude material was purified by chromatography on silica gel (500 g). The column was packed in 40:25 MeOH : CHCl<sub>3</sub> and eluted with 40:25 MeOH : CHCl<sub>3</sub> and then 40:25:10 MeOH : CHCl<sub>3</sub> : NH<sub>4</sub>OH. The fractions collected were analyzed by thin layer chromatography and the product containing fractions were combined and concentrated *in vacuo* (the evaporation was assisted by the addition of *iso*-propanol in order to azeotrope the residual water). The material was vacuum dried at ambient temperature for 48 hours to give N<sup>4</sup>-spermine cholesteryl carbamate (4g, 6.5 mmol, 67% yield).

(B) N<sup>4</sup>-(N'-cholesteryl carbamate glycineamide)-spermine (amphiphile No. 91)

N-t-BOC-glycine-N-hydroxysuccinimide ester (0.5 g, 1.83 mmol) was added to a solution of diCbz-spermine•2HCl (1.0 g, 1.94 mmol) and N,N-

diisopropylethylamine (0.3 mL, 1.72 mmol) in 65/ 25/ 4 chloroform/ methanol/ water (50 mL). The solution was stirred overnight at room temperature. Analysis of the reaction by TLC (20% methanol/ chloroform) indicated the presence of a new spot. The reaction was washed first with  
5 1M NaOH (10 mL) then with H<sub>2</sub>O (10 mL). The organic layer was separated, dried over sodium sulfate, vacuum filtered, and reduced *in vacuo* to an oil. The crude material was purified by flash column chromatography (85 g silica gel) eluting with 20% methanol/ chloroform. The desired product was isolated and characterized by <sup>1</sup>H NMR as N<sup>1</sup>,N<sup>12</sup>-diCbz-N<sup>4</sup>-  
10 (N'-t-BOC-glycineamide)-spermine (402 mg, 0.65 mmol, 35%).

Benzyl chloroformate (100 mg, 0.58 mmol) was added to a solution of N<sup>1</sup>,N<sup>12</sup>-diCbz-N<sup>4</sup>-(N'-t-BOC-glycineamide)-spermine (220 mg, 0.354 mmol) and triethylamine (4 drops) in methylene chloride (20 mL). The reaction was stirred overnight at room temperature. Analysis of the reaction by TLC  
15 (20% methanol/ chloroform) indicated the presence of a new, higher running spot. The reaction was quenched by the addition of 1M HCl (5 mL). The organic layer was isolated, washed with H<sub>2</sub>O (5 mL), dried over sodium sulfate, filtered, and reduced *in vacuo*.

The resulting crude material was dissolved in chloroform (30 mL)  
20 and anhydrous HCl gas was bubbled through the solution for 2 hours. Analysis of the reaction by TLC (10% methanol/ chloroform) indicated the complete disappearance of the starting material. The reaction was purged with dry nitrogen, and washed with 1M NaOH (2 x 10 mL) and dH<sub>2</sub>O (10 mL). The organic layer was isolated, dried over sodium sulfate, filtered, and  
25 reduced *in vacuo* to give N<sup>1</sup>,N<sup>9</sup>,N<sup>12</sup>-triCbz-N<sup>4</sup>-glycineamide-spermine (219 mg, 0.33 mmol, 93% yield for two steps).

Cholesteryl chloroformate (148 mg, 0.33 mmol) was added to a solution of N<sup>1</sup>,N<sup>9</sup>,N<sup>12</sup>-triCbz-N<sup>4</sup>-glycineamide-spermine (219 mg, 0.33 mmol) and triethylamine (0.3 mL, 2.15 mmol) in methylene chloride (30 mL). The reaction was stirred at room temperature for 3 hours. The  
5 reaction was washed with H<sub>2</sub>O (10 mL). The organic layer was separated, dried over sodium sulfate, filtered, and reduced *in vacuo*. The crude material was purified by flash column chromatography (30 g silica gel) eluting with 65% ethyl acetate/ hexanes. The desired product was isolated and characterized by <sup>1</sup>H NMR as N<sup>1</sup>,N<sup>9</sup>,N<sup>12</sup>-triCbz-N<sup>4</sup>-(N'-cholesteryl  
10 carbamate glycineamide)-spermine (221 mg, 0.2 mmol, 62% yield).

N<sup>1</sup>,N<sup>9</sup>,N<sup>12</sup>-tri-Cbz-N<sup>4</sup>-(N'-cholesteryl carbamate glycineamide)-spermine (221 mg, 0.2 mmol) was stirred with 10% Pd/C (50 mg) in glacial acetic acid (10 mL) under a hydrogen atmosphere for 2.5 hours. Analysis of the reaction by TLC (65% ethyl acetate/ hexanes) indicated the complete  
15 disappearance of the starting material. The flask was purged with nitrogen and the catalyst was removed by vacuum filtration through filter paper rinsing with 10% acetic acid/ ethyl acetate (20 mL). The filtrate was reduced *in vacuo* to an oil which was dissolved in 10% methanol/ chloroform (100 mL) and washed with 1M NaOH (20 mL) and H<sub>2</sub>O (15 mL). The organic  
20 layer was separated, dried over sodium sulfate, filtered, and reduced *in vacuo*. The isolated product was characterized by <sup>1</sup>H NMR as N<sup>4</sup>-(N'-cholesteryl carbamate glycineamide)-spermine (128 mg, 0.19 mmol, 95% yield).

(C) Synthesis of N<sup>4</sup>-spermidine-2,3-dilauryloxypropylamine, amphiphile No.  
25 94.

2,3 Dimyristoylglycerol (600 mg, 1.4 mmol) was dissolved in pyridine and the solution cooled to 0°C. The solution was stirred under a nitrogen

atmosphere and p-toluenesulfonyl chloride (300 mg, 1.57 mmol) was added. The solution was allowed to warm to room temperature and was then stirred overnight at ambient temperature. To the solution was added hydrochloric acid (2.5M, 20 mL) and the solution was extracted three times  
5 with methylene chloride (25 mL). The combined organic extracts were dried over sodium sulfate, filtered and concentrated in *vacuo* to give a crude oil. The oil was purified by flash chromatography (50g of silica gel, 60Å) eluting with 5% ethyl acetate / hexane. The oil obtained by flash chromatography was dried under high vacuum at ambient temperature to give 2,3-  
10 Dimyristoylglycerol-tosylate (630 mg, 77% yield).

2,3-Dimyristoylglycerol-tosylate (300 mg, 0.51 mmol) and N<sup>1</sup>N<sup>8</sup>-diCbz-spermidine (1.5g, 3.6 mmol) were dissolved in toluene (15 mL). The solution was stirred under a nitrogen atmosphere and heated at reflux (110°C). The reaction was heated for 5 days at reflux temperature. The  
15 reaction was cooled to room temperature and then filtered through Whatman #1 filter paper. The filtrate was concentrated in *vacuo*. The residue was dissolved in chloroform (50 mL) and washed with sodium hydroxide solution (1 M, 10 mL) and water (10 mL). The organic fraction was dried over sodium sulfate, filtered and concentrated in *vacuo*. The  
20 crude material was purified by flash chromatography (30g silica gel, 60Å) eluting with 5% methanol / chloroform. The product containing fractions were concentrated in *vacuo*. The material was purified by a second flash chromatography column (20 g silica, 60Å) eluting with 50% ethyl acetate / hexane. Chromatography gave, after drying the product under high  
25 vacuum at ambient temperature, N<sup>4</sup>-(N<sup>1</sup>N<sup>8</sup>)-diCbz-spermidine-2,3-dilauryloxypropylamine, as an oil (142 mg, 35% yield).



$N^4(N^1,N^8)$ -diCbz-spermidine-2,3-dilauryloxypropylamine (142 mg, 0.18 mmol) in glacial acetic acid (5 mL) was stirred with 10% Pd/C (50 mg) under a hydrogen atmosphere, for 2 hours. The catalyst was removed by vacuum filtration through Whatman #1 filter paper. The catalyst was  
5 washed with ethyl / acetate hexane (10%, 10 mL). The filtrate was concentrated in *vacuo* and dried for 2 hours under high vacuum. To the residue was added sodium hydroxide solution (1 M, 8 mL) and the solution was extracted three times with methanol / chloroform (10%, 20 mL). The combined organic extracts were dried over sodium sulfate, filtered and  
10 concentrated in *vacuo* to give after drying under high vacuum  $N^4$ -spermidine-2,3-dilauryloxypropylamine (52 mg, 52% yield).

(D) Synthesis of  $N^4$ -spermine-2,3-dilauryloxypropylamine, amphiphile No. 102

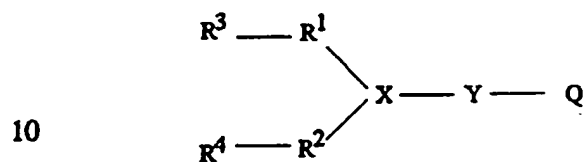
$N^1,N^{12}$ -diCbz-spermine (0.87g, 1.85 mmol) and 2,3-dimyristoylglycerol-tosylate (280mg, 0.48 mmol) were dissolved in toluene (25 mL) and heated at reflux temperature (110°C) for 3 days. The solution was concentrated in *vacuo* and the resulting material was purified by flash chromatography (30g silica gel, 60Å) eluting with 10% methanol / chloroform. The material isolated was dissolved in methanol / chloroform  
20 (10%, 85 mL) and washed twice with sodium hydroxide solution (1 M, 15 mL) and water (10 mL). The organic fraction was dried over sodium sulfate, filtered and concentrated in *vacuo*. The material was dried under high vacuum overnight, at ambient temperature, to yield  $N^4(N^1,N^{12}$ -diCbz-spermine)-2,3-dilauryloxypropylamine (180 mg, 43 % yield).

25  $N^4(N^1,N^{12}$ -diCbz-spermine)-2,3-dilauryloxypropylamine (180 mg, 0.2 mmol) in glacial acetic acid (10 mL) was stirred with 10% Pd/C (50 mg) under a hydrogen atmosphere, for 3 hours. The catalyst was removed by

vacuum filtration through Whatman #1 filter paper. The catalyst was washed with ethyl / acetate hexane (10%, 30 mL). The filtrate was concentrated in *vacuo* and dried for 2 hours under high vacuum. To the residue was added methanol / chloroform (10%, 85 mL) and the organic  
5 layer was washed twice with sodium hydroxide solution (1 M, 15 mL) and water (10 mL). The organic fraction was dried over sodium sulfate, filtered and concentrated in *vacuo* to give after drying under high vacuum N<sup>4</sup>-spermine-2,3-dilauryloxypropylamine (50 mg, 40% yield).

Claims :

- 5 1. A cationic amphiphile compound characterised in that it corresponds to the following general formula:



(V)

wherein

- 15  $R^1$  and  $R^2$  each independently represents -N(H)-, alkylamine or polyalkylamine, provided that not both represent -N(H)-;

$R^3$  and  $R^4$  each independently represents H, an optionally derivatized amino acid or a saturated or unsaturated aliphatic group;

20

X represents C or N;

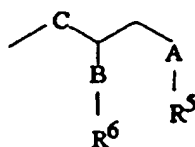
Y, if present, represents a linking group;

25

and

Q represents -Z or

30



wherein

Z represents alkylamine, dialkylamine, which may have different alkyl groups, or a steroid;

5

A and B each independently represents O, N or S ;

C represents  $-C(H_2)-$ ,  $-C(O)-$  or  $-C(S)-$  ;

10

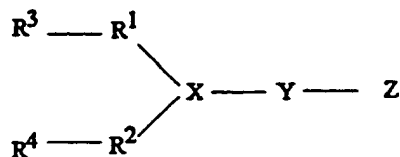
and

$R^5$  and  $R^6$  each independently represents a saturated or unsaturated alkyl or acyl group.

15

2. A compound as claimed in claim 1 wherein it corresponds to one of the following general formulae:

20



(I)

wherein

25

Z represents a steroid;

X represents C or N;

Y represents a short linking group or is absent;

30

$R^1$  and  $R^2$  each independently represents  $-N(H)-$ , alkylamine or polyalkylamine, provided that not both represent  $-N(H)-$ ;

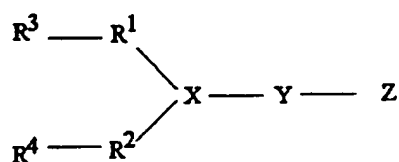
and

R<sup>3</sup> and R<sup>4</sup> each independently represents H or a saturated or unsaturated aliphatic group;

5

or

10



(II)

wherein

15

Z, X, R<sup>1</sup> and R<sup>2</sup> are as defined above in connection with general formula (I);

Y represents a linking group or is absent;

20

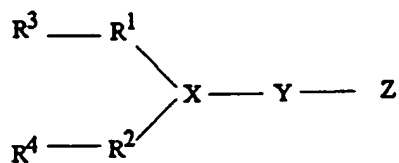
and

R<sup>3</sup> and R<sup>4</sup> each independently represents an optionally derivatized amino acid, H or alkyl;

25

or

30



(III)

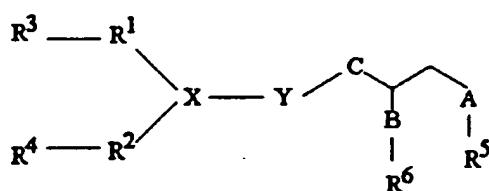
wherein

X, Y, R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup> and R<sup>4</sup> are as defined above in connection with general formula (I);

and

Z represents alkylamine or dialkylamine, which may have different alkyl groups;

or



(IV)

wherein

X, R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup> and R<sup>4</sup> are as defined above in connection with general formula (I);

A and B each independently represents O, N or S;

C represents -C(H<sub>2</sub>)-, -C(O)- or -C(S)-;

Y, if present, represents -(H)N(C(O))- or -O(C(O))-;

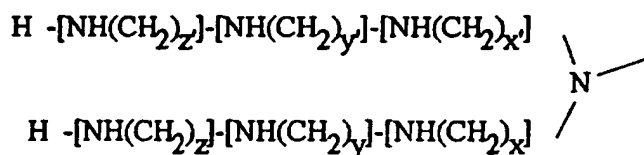
and

R<sup>5</sup> and R<sup>6</sup> each independently represents a saturated or unsaturated alkyl or acyl group.

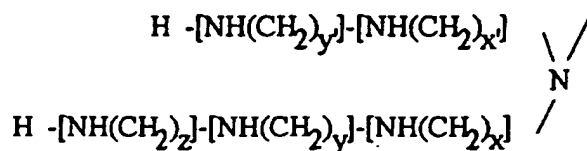
3. A compound as claimed in claim 1 or claim 2, general formula (I):

wherein Z represents a 3-sterol linked via its 3-O- group or via N in replacement thereof;

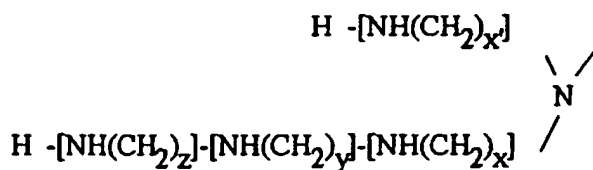
or wherein Z - Y - is connected to



or



or



the total number of nitrogen and carbon atoms in each (poly)alkylamine group being less than about 30 and each  $x, x', y, y', z$  and  $z'$  being an integer greater than 1;

5



15

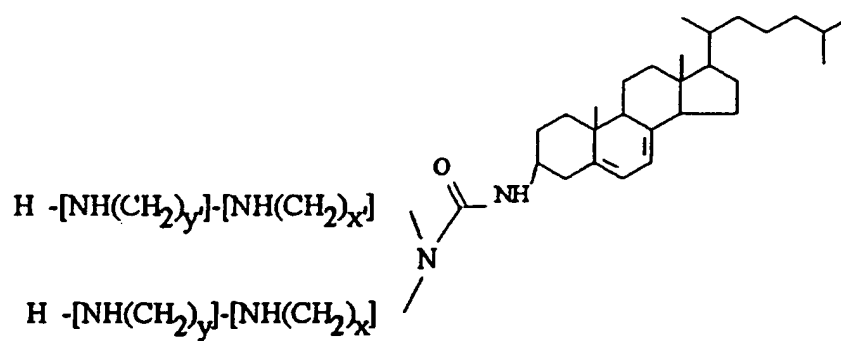


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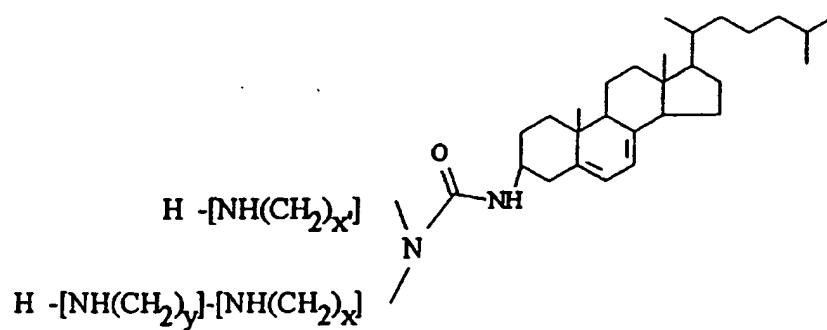




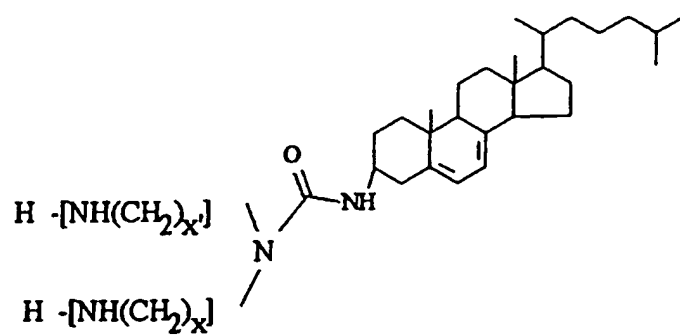
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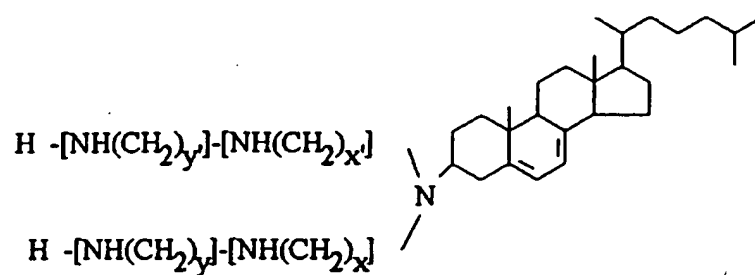


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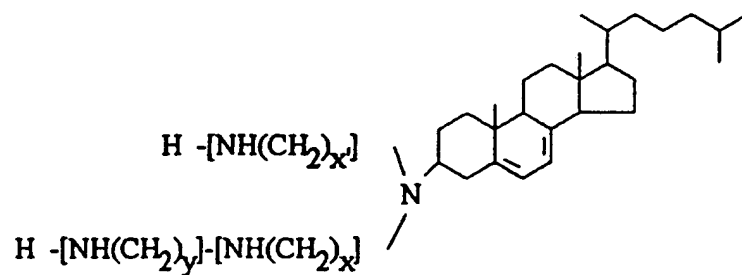
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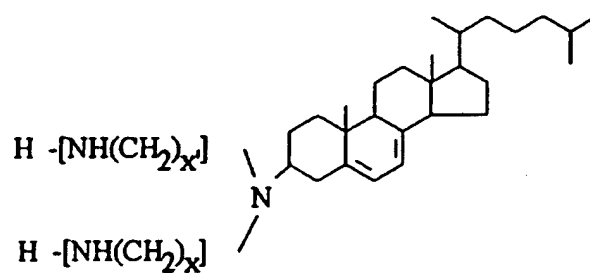
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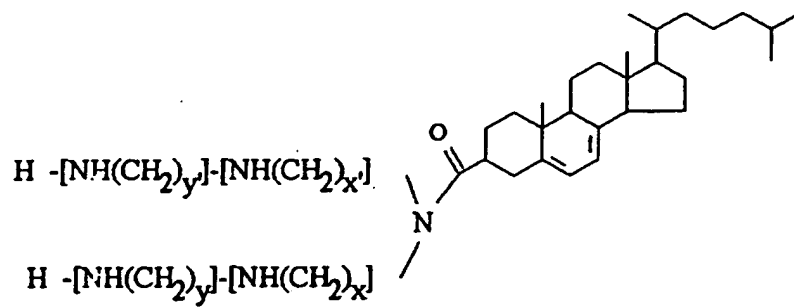
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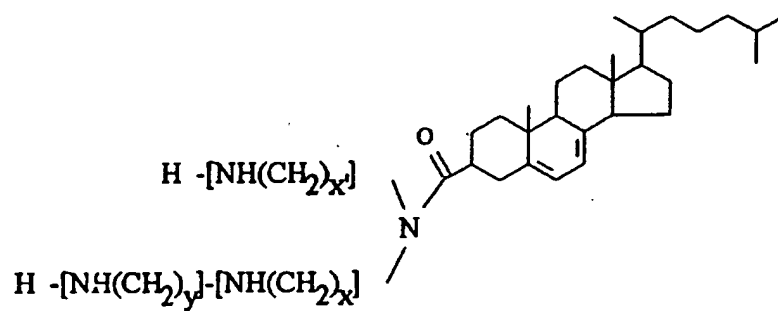


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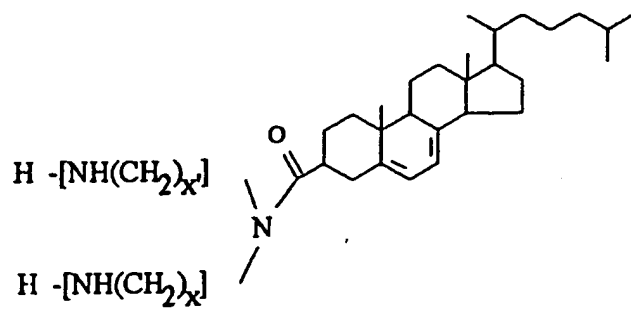
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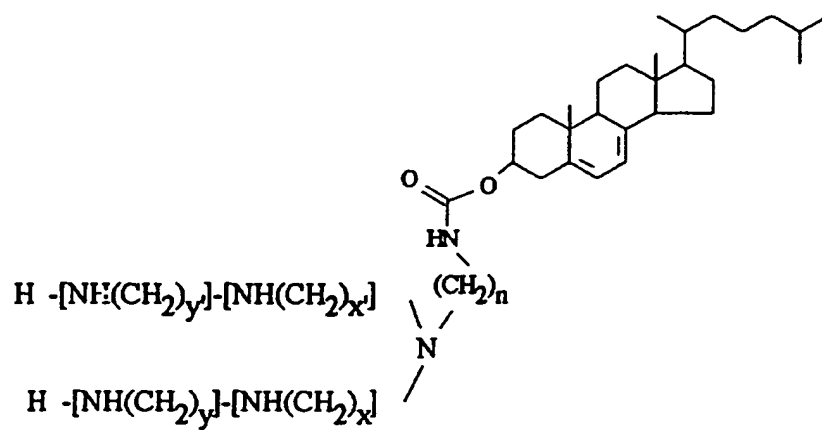


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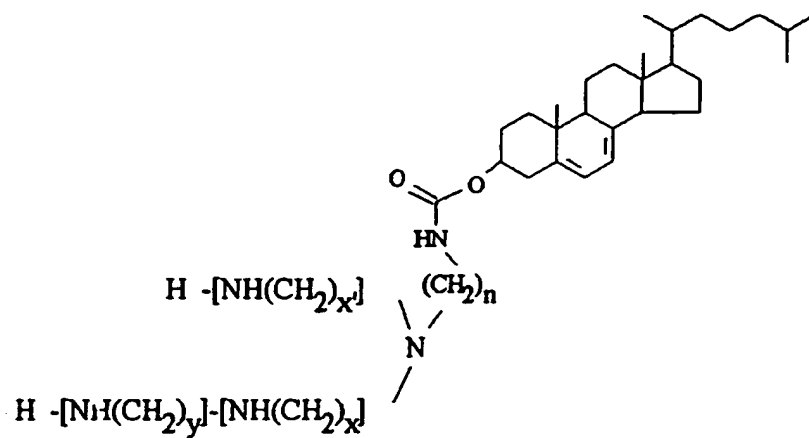
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10

or

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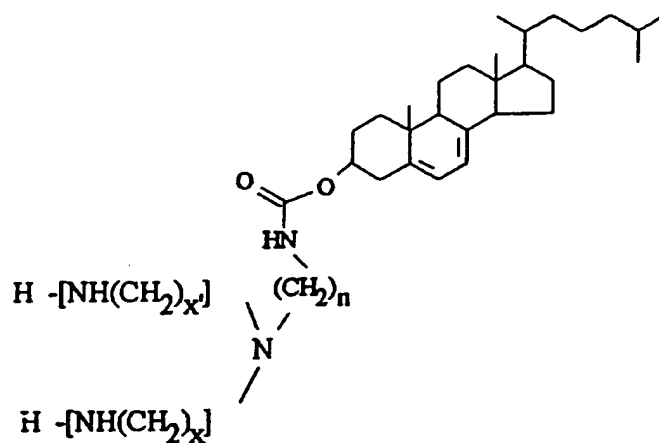


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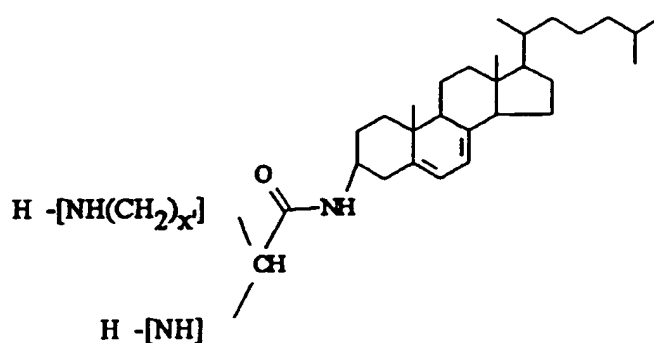
25

or

30



or



25 the total number of nitrogen and carbon atoms in each (poly)alkylamine group being less than about 30 and each of  $x$ ,  $x'$ ,  $y$  and  $y'$  being an integer greater than 1, except in the last formula when  $x'$  may also represent 1, preferably at least one being 3 and at least one being 4; optionally the double bond at C5 and/or C7 in the steroid ring being hydrogenated; and  $n$  being an integer of from 1 to 4;

30 or wherein it is numbered 53, 61, 65, 67, 69, 70, 71, 72, 75, 78, 79, 81, 82, 83, 84, 86, 88, 90, 92, 96, 101, 104, 106 or 107 herein.

4. A compound as claimed in claim 1 or claim 2, general formula (II):

wherein no more than one atom of the said linking group Y forms a bond with both X and Z, Y preferably being carbonyl;

or wherein the said linking group Y is an acylated amino acid, the carbonyl from the amino acid being attached to X and the carbonyl from the N-acyl moiety being attached to an oxygen or nitrogen of the steroid Z, Y preferably being an N-acetylglycine or N-acylserine;

or wherein Z represents a 3-sterol linked via its 3-O- group or via N in replacement thereof, Z preferably being cholesterol;

or wherein two or more nitrogens in the R<sup>1</sup> and/or R<sup>2</sup> (poly)alkylamine groups are separated by one or more combinations of 3 and of 4 carbons;

or wherein it is numbered 87, 91, 93, 95, 97, 99, 100 or 103 herein.

5. A compound as claimed in claim 1 or claim 2, general formula (III):

wherein

Z represents a dialkylamine;

Y represents a linking group, preferably carbonyl or methylene, no more than one atom thereof forming a bond with both X and Z;

X represents carbon;

R<sup>3</sup> and R<sup>4</sup> represent hydrogen,

5 R<sup>1</sup> represents an alkylamine; and

R<sup>2</sup> represents -N(H)- ;

10 or wherein it is N,N-dioctadecyl lysine amide, N,N-didecyl lysine amide, N,N-didodecyl lysine amide or N<sup>1</sup>, N<sup>1</sup>-dioctadecyl-1,2,6 -triaminohexane;

or wherein

15 Z represents an alkylamine or a dialkylamine;

Y represents a linking group preferably carbonyl or methylene, no more than one atom thereof forming a bond with both X and Z;

20 X represents carbon;

R<sup>3</sup> and R<sup>4</sup> represent hydrogen;

25 R<sup>1</sup> represents a polyalkylamine, preferably two or more nitrogens being separated by one or more combinations of 3 and of 4 carbons; and

R<sup>2</sup> represents -N(H)- ;

30

or wherein

Z represents an alkylamine or a dialkylamine;

5 Y represents a linking group, preferably carbonyl or methylene, no more than one atom thereof forming a bond with both X and Z;

X represents carbon or nitrogen;

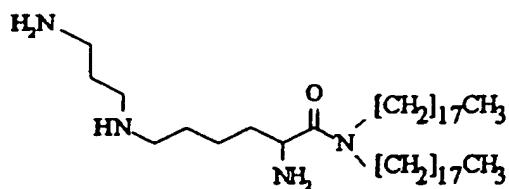
10 R<sup>3</sup> and R<sup>4</sup> represent hydrogen; and

R<sup>1</sup> and R<sup>2</sup> each independently represents an alkylamine or polyalkylamine, preferably two or more nitrogens being separated by one or more combinations of 3 and of 4 carbons;

15

or wherein it corresponds to one of the following formulae:

20

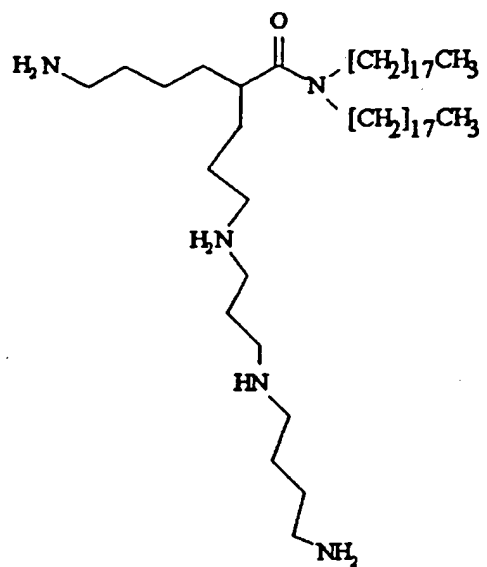


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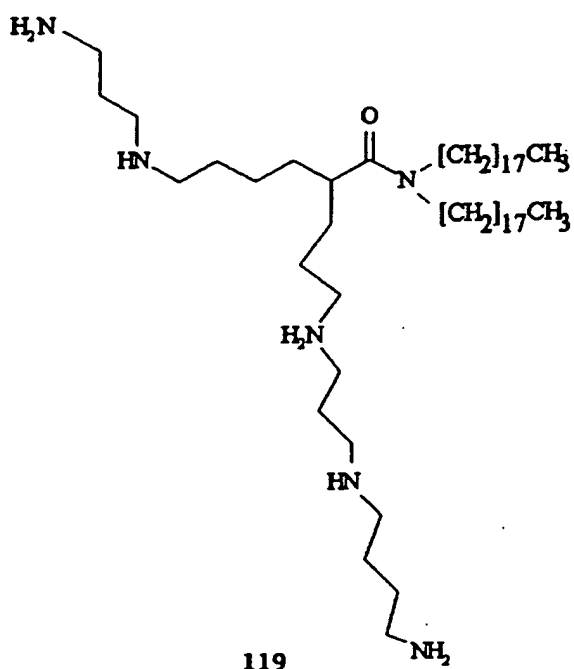
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or

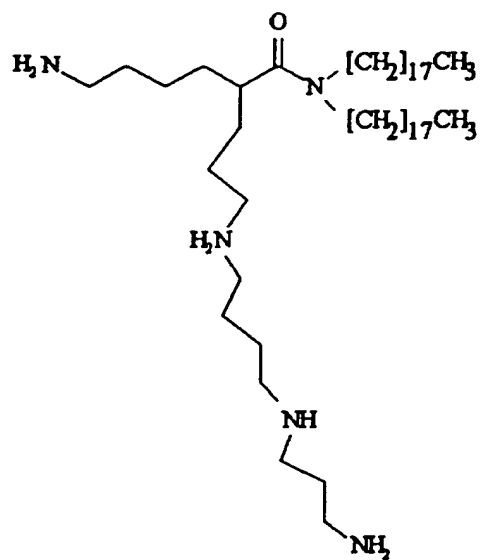




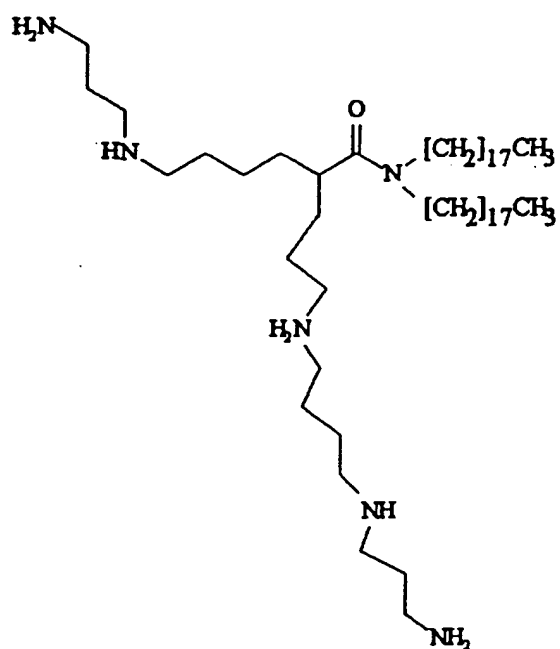
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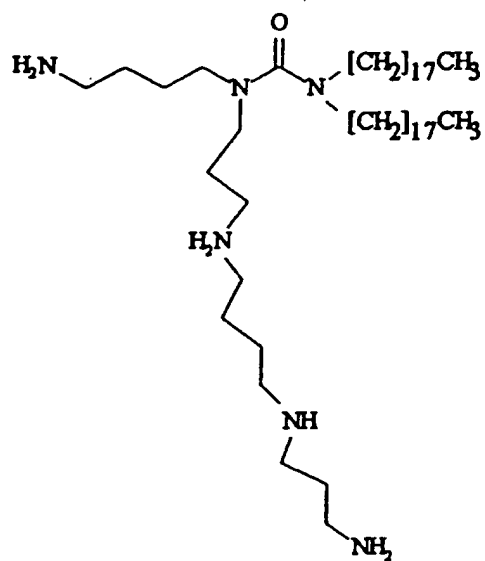
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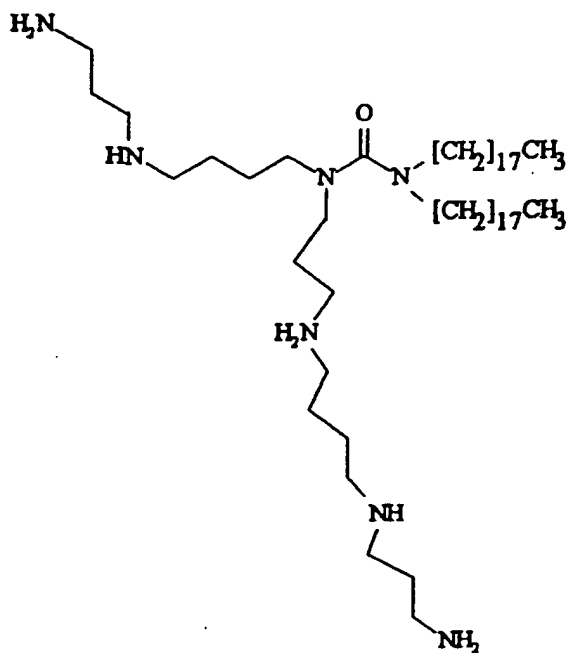
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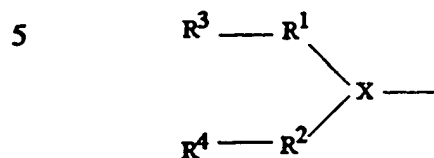


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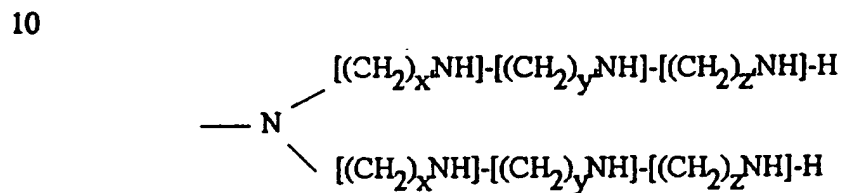


6. A compound as claimed in claim 1 or claim 2, general formula (IV):

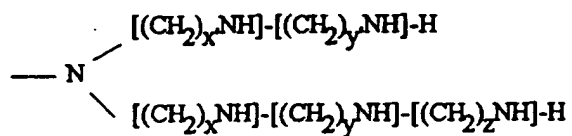
wherein



represents

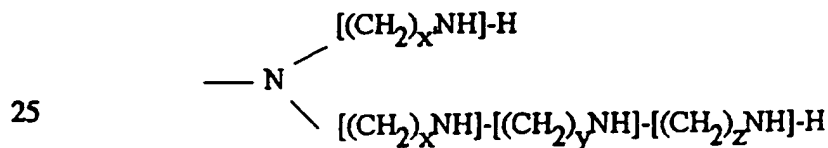


15 or



20

or



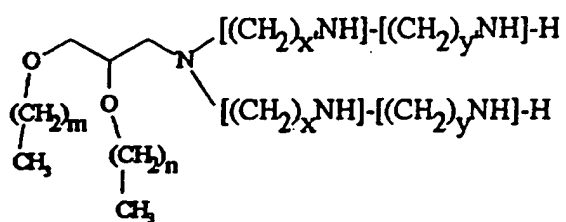
the total number of nitrogen and carbon atoms in each (poly)alkylamine group being less than about 30 and each of x, x', y, y', z and z' being an integer greater than 1;

30

or wherein it corresponds to one of the following general formulae:

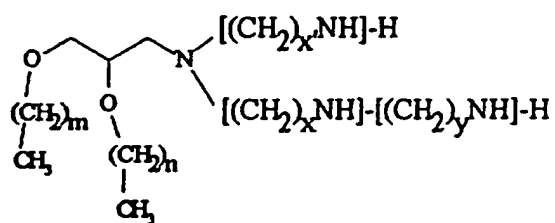


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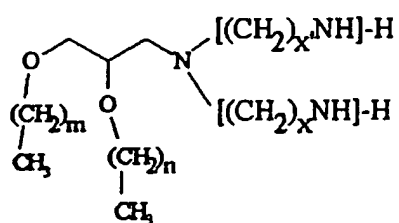
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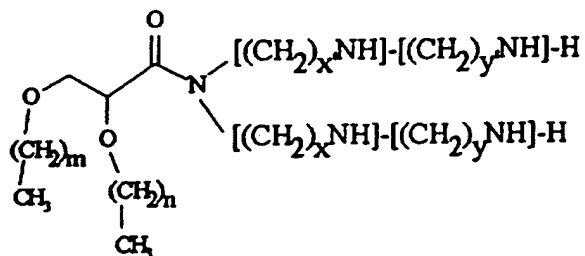
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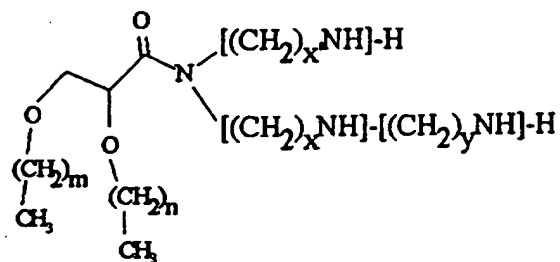
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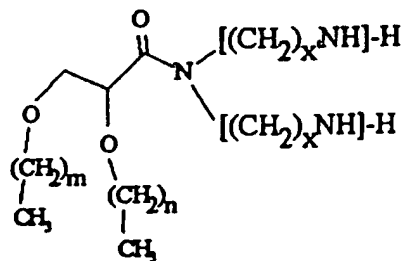
30



or



or



30 the total number of nitrogen and carbon atoms in each (poly)alkylamine group being less than about 30, each of x, x', y and y' being an integer greater than 1, preferably

at least one being 3 and at least one being 4, and m and n each being between about 7 and about 29;

5 or wherein it is numbered 64, 76, 85, 89, 94, 98, 102, 105, 110 or 111 herein.

7. A pharmaceutical composition characterised in that it comprises a complex comprising:

- 10 (1) a cationic amphiphile compound as claimed in any of claims 1 to 6;
- (2) a biologically active molecule selected from ribosomal RNA, an antisense polynucleotide of RNA or DNA, a ribozyme and a polynucleotide of genomic DNA, cDNA, or mRNA encoding a therapeutically useful protein;

15

and, optionally,

- (3) a co-lipid;

20 and also, optionally, an excipient selected from lactose, trehalose, sucrose, mannitol, maltose and galactose, which composition may be in lyophilized form.

8. A composition as claimed in claim 7 wherein the biologically active molecule (2) thereof is a DNA that comprises (i) a nucleotide sequence encoding a therapeutically useful polypeptide and (ii) one or more regulatory elements capable of facilitating expression of the encoding sequence in a cell of a patient, preferably the encoding nucleotide sequence coding for a polypeptide having an amino acid sequence sufficiently duplicative of that of CFTR to allow possession of the biological property of epithelial cell anion channel regulation.
- 25

30

9. A gene therapy composition for a disease state associated with inflammation characterized in that it comprises a DNA molecule capable of entering cells of tissue



affected by the disease state, the DNA molecule comprising: (1) a transgene capable of providing treatment for the disease state, preferably selected from CFTR, p53, alpha-1 antitrypsin, TIMP-1 and TIMP-2; and, upstream therefrom, (2) a nucleotide sequence corresponding to that of an RNA polymerase promoter for a cytokine gene, preferably selected from interleukin 2, interleukin 8, interleukin 1, interleukin 11, interleukin 6, endothelin -1, monocyte chemoattractant protein -1, IL-1ra and GM-CSF, expression of which is upregulated at the transcriptional level by inflammation; expression of the transgene preferably being upregulated by tumor necrosis factor; which DNA molecule may be complexed with a cationic amphiphile compound as claimed in any of claims 1 to 6, preferably corresponding to general formula (I), more preferably a compound numbered 53, 67, 75, 78 or 90 herein.

10. A gene therapy composition for intravenous treatment of a patient's heart characterized in that it comprises : (1) a DNA molecule including an encoding sequence for a therapeutic protein; and (2) a cationic amphiphile compound as claimed in any of claims 1 to 6, preferably corresponding to general formula (I) or (II), more preferably a compound numbered 53, 67, 75, 78 or 90 herein; the composition being provided as a formulation such that the molar ratio of cationic amphiphile compound : DNA (measured by molarity of nucleotide) causes the cationic amphiphile compound /DNA complexes in solution to have a positive zeta potential, preferably about +30 mV.

11. A composition as claimed in claim 10 wherein the cationic amphiphile compound is N<sup>4</sup>-spermidine cholesteryl carbamate (53) and the molar ratio of cationic amphiphile compound : DNA is about 1.25:1, preferably the composition being formulated by mixing: (1) a first solution consisting of unprotonated free base form of N<sup>4</sup>-spermidine cholesteryl carbamate, zwitterionic DOPE and water, the molar ratio of cationic amphiphile compound : DOPE being 1:1, and (2) a second solution consisting of the sodium salt of the DNA in water.

12. An episomal plasmid characterized in that it is capable of being maintained and replicated in the nucleus of a human cell and comprises a polydeoxyribonucleotide

containing more than one origin of replication that is capable of being recognized by human DNA polymerase and that facilitates interaction of the polymerase with the plasmid, preferably the nucleotide sequence defining the origins of replication being a continuous sequence of between about 4.8 and about 9.6 kb in length, preferably an origin of replication being that found within the human  $\beta$ -globin gene, the mouse DHFR gene or, more preferably, within a 2.4 kb HindIII-XhoI fragment that is present immediately 5' to exon 1 of the human c-myc gene, the plasmid preferably having a size of about 18 kb and preferably having the characteristics of pMyc4-CFTR.

10

13. A therapeutic transfection plasmid characterized in that it consists essentially of pCF1 or pCF2, and a transgene.

15

14. A method of providing a program of gene therapy to a patient minimizing the frequency of administrations characterized in that it comprises treating a patient using a therapeutic composition comprising a plasmid as claimed in claim 12, the plasmid comprising a transgene that is capable of providing treatment for a disease state, preferably the plasmid containing, as transgene, an encoding sequence for human cystic fibrosis transmembrane conductance regulator, the therapeutic composition being suitable for treating the lung of a cystic fibrosis patient, the performance of the plasmid being enhanced by inclusion therein of one or more selected from the cytomegalovirus promoter, the cytomegalovirus enhancer, an intron placed upstream from the encoding sequence for CFTR, the bovine growth hormone polyadenylation signal and the kenamycin resistance transposon Tn903.

25

30

15. A method of providing gene therapy to a patient suffering from a disease state involving an inflammatory condition characterized in that it comprises contacting an affected tissue with a therapeutic composition comprising a DNA molecule capable of entering cells of the tissue, the DNA molecule including an encoding sequence for a transgene capable of providing treatment for the disease state, the DNA molecule also including the improvement of a nucleotide sequence, itself comprising an RNA polymerase promoter derived from a gene for a cytokine that is upregulated by

inflammation, and that is appropriately positioned in the DNA molecule so that expression from the transgene is placed under the control thereof, preferably the cytokine RNA polymerase promoter included in the DNA molecule being derived from a cytokine selected from interleukin 2, interleukin 8, interleukin 1, interleukin 11, interleukin 6, endothelin-1, monocyte chemoattractant protein-1, IL-1ra and GM-CSF.

16. A method of providing gene therapy to the heart of a patient characterized in that it comprises intravenous administration of a therapeutic composition as claimed in claim 10.

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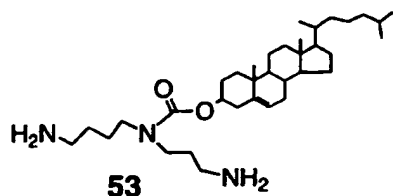
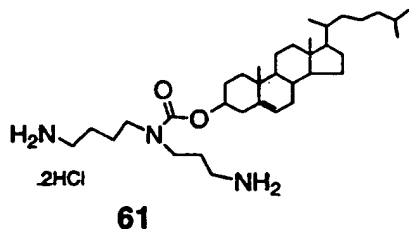
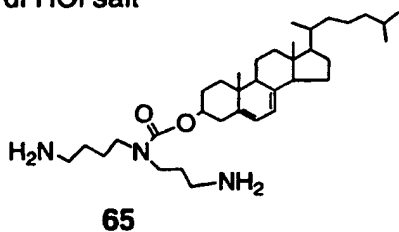
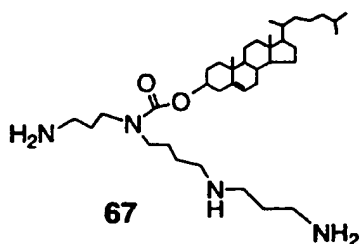
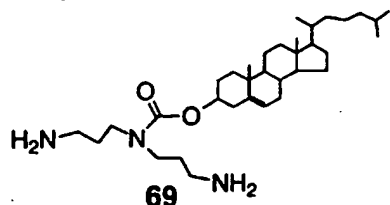
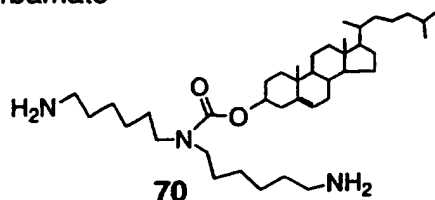
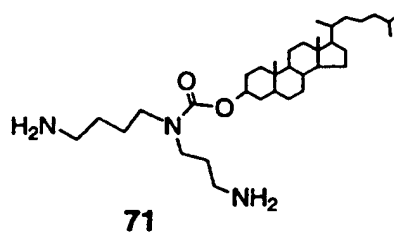
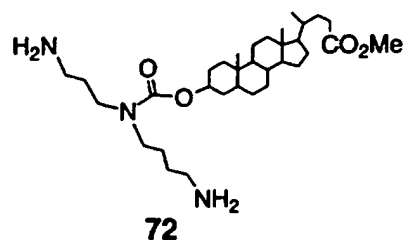
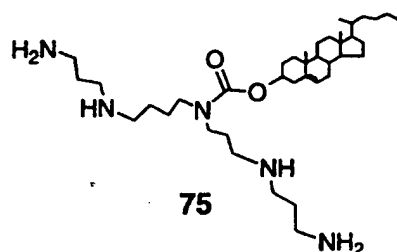
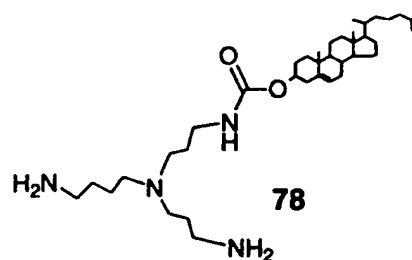
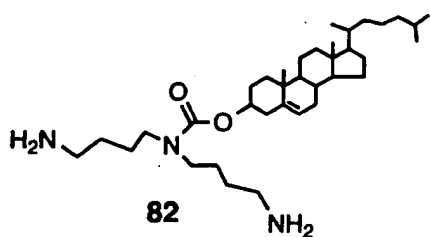
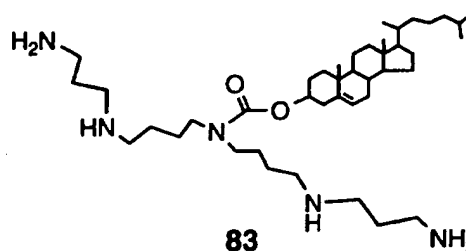
N<sup>4</sup>-spermidine cholesteryl carbamateN<sup>4</sup>-spermidine cholesteryl carbamate  
di HCl saltN<sup>4</sup>-spermidine-7 dehydro cholesteryl  
carbamateN<sup>4</sup>-spermine cholesteryl carbamateN,N bis (3-aminopropyl) cholesteryl  
carbamateN,N bis (6-aminohexyl) chol st ryl  
carbamateN<sup>4</sup>-spermidine dihydrocholesteryl carbamateN<sup>4</sup>-spermidine lithocholic carbamate  
methyl esterN<sup>1</sup>,N<sup>8</sup>-Bis (3-aminopropyl)- N<sup>4</sup> -  
spermidine cholesteryl carbamateN(N<sup>4</sup>-3-aminopropylspermidine)  
cholesteryl carbamate

FIG. 1 A

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N,N-Bis(4-aminobutyl) cholesteryl carbamate



N,N-Bis(N'-3-aminopropyl-N''-4-aminobutyl) cholesteryl carbamate

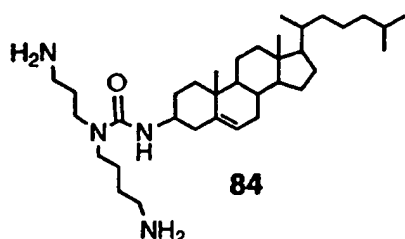
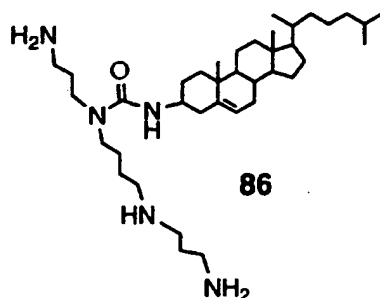
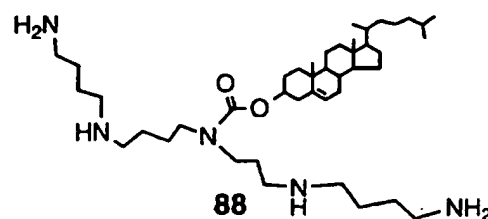
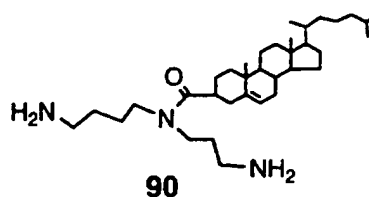
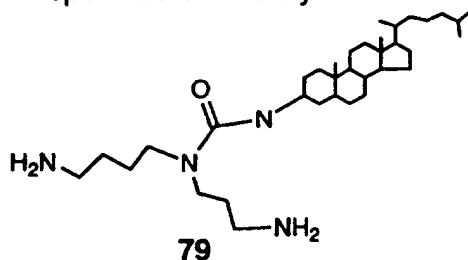
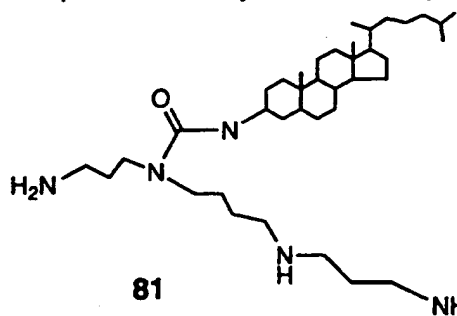
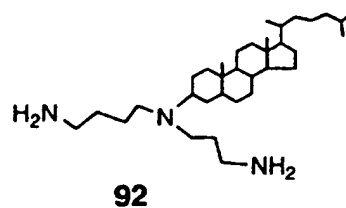
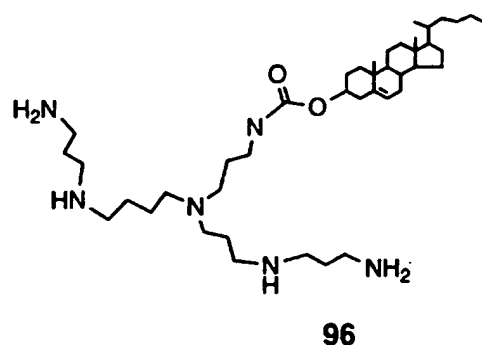
N<sup>4</sup>-spermidine cholesteryl ureaN<sup>4</sup>-spermine cholesteryl ureaN<sup>4</sup> Spermidine cholesteryl carboxamideN<sup>4</sup>-spermidine dihydro cholesteryl ureaN<sup>4</sup>-sp min dihydro cholest ryl ureaN-[N<sup>1</sup>,N<sup>4</sup>,N<sup>8</sup>-Tris (3-aminopropyl) spermidine] cholesteryl carbamate

FIG. 1B

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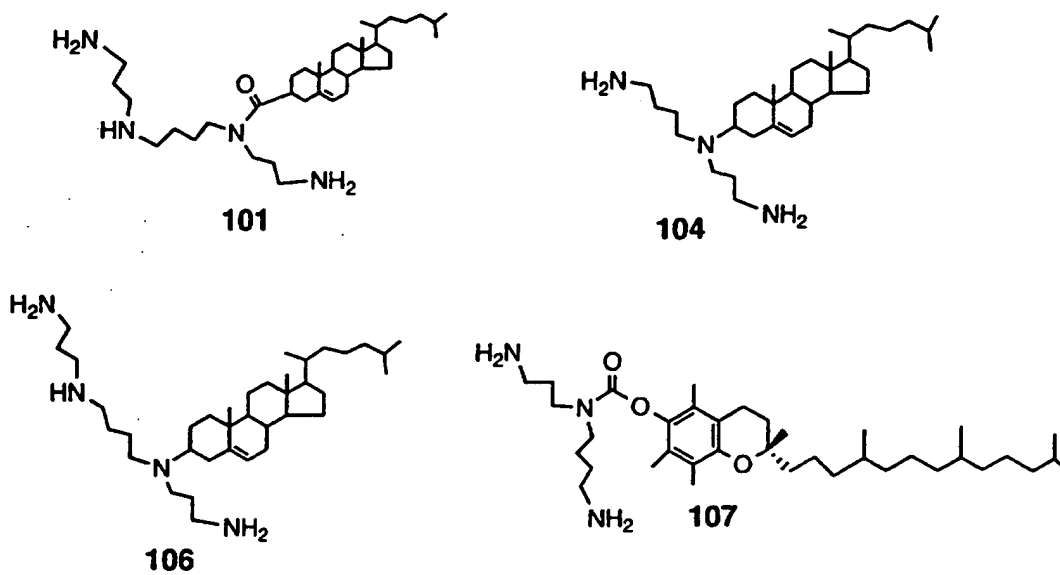


FIG. 1B

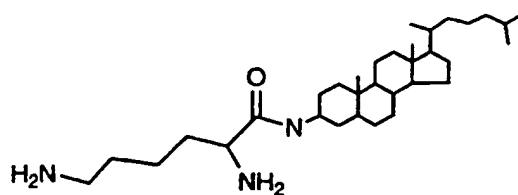
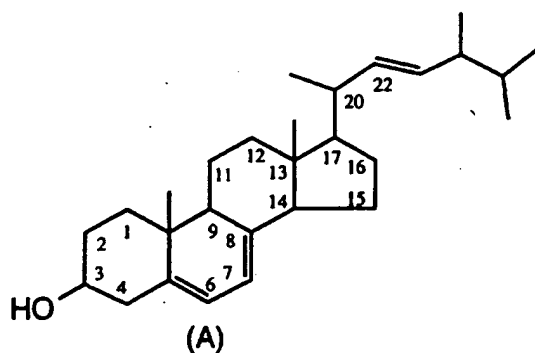
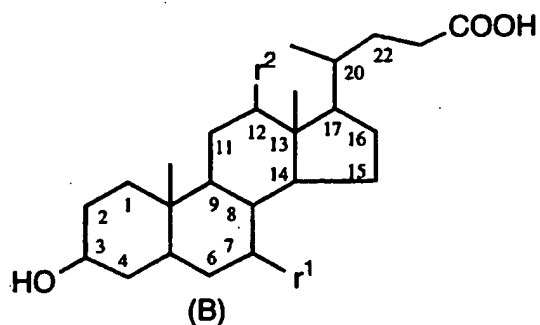


FIG. 1C

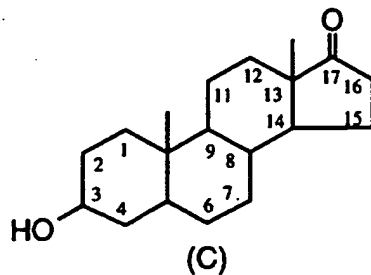
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Ergosterol (double bonds as shown)  
 Ergosterol B1 ( $\Delta 8, 9; \Delta 14, 15; \Delta 22, 23$ )  
 Ergosterol B1 ( $\Delta 6, 7; \Delta 8, 14; \Delta 22, 23$ )  
 Ergosterol B1 ( $\Delta 7, 8; \Delta 14, 15; \Delta 22, 23$ )  
 Lumisterol ( $9\beta$ -H isomer of ergosterol)



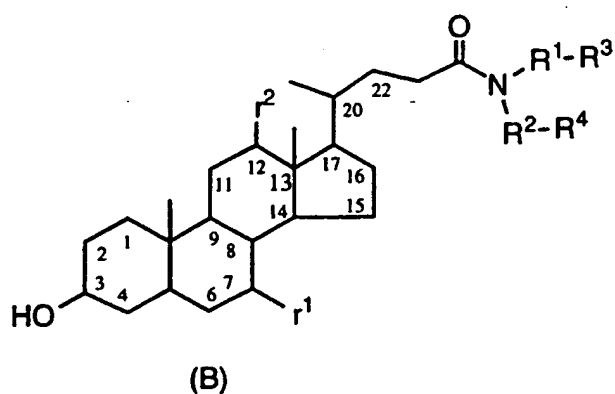
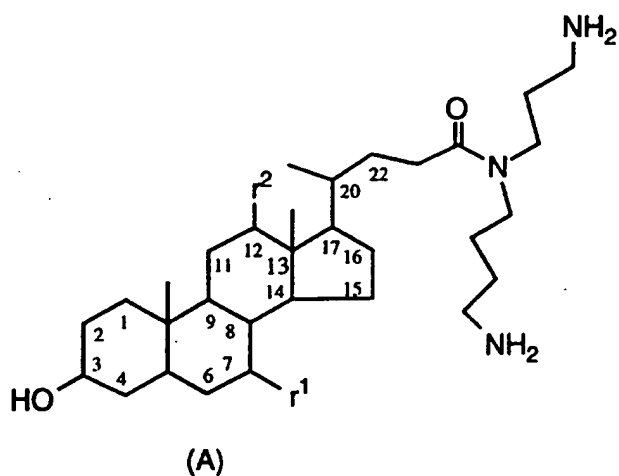
Cholic Acid  $r^1, r^2 = \text{OH}$   
 Desoxycholic Acid  $r^1 = \text{H}, r^2 = \text{OH}$   
 Chenodesoxycholic Acid  $r^1 = \text{OH}, r^2 = \text{H}$   
 Lithocholic Acid  $r^1, r^2 = \text{H}$



Androsterone

FIG. 2

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Cholic Acid Derivative  $r^1, r^2 = \text{OH}$   
Desoxycholic Acid Derivative  $r^1 = \text{H}, r^2 = \text{OH}$   
Chenodesoxycholic Acid Derivative  $r^1 = \text{OH}, r^2 = \text{H}$   
Lithocholic Acid Derivative  $r^1, r^2 = \text{H}$

FIG. 3



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Transacylation Reaction.

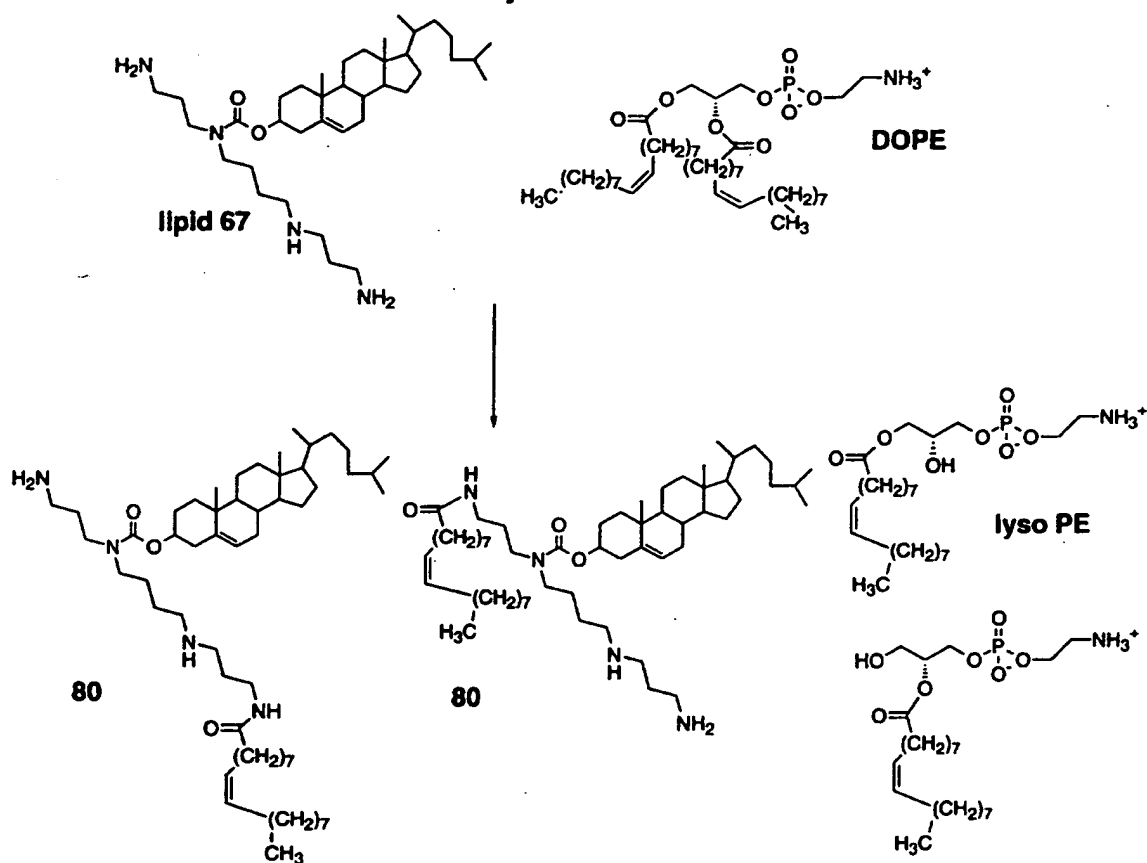


FIG. 4

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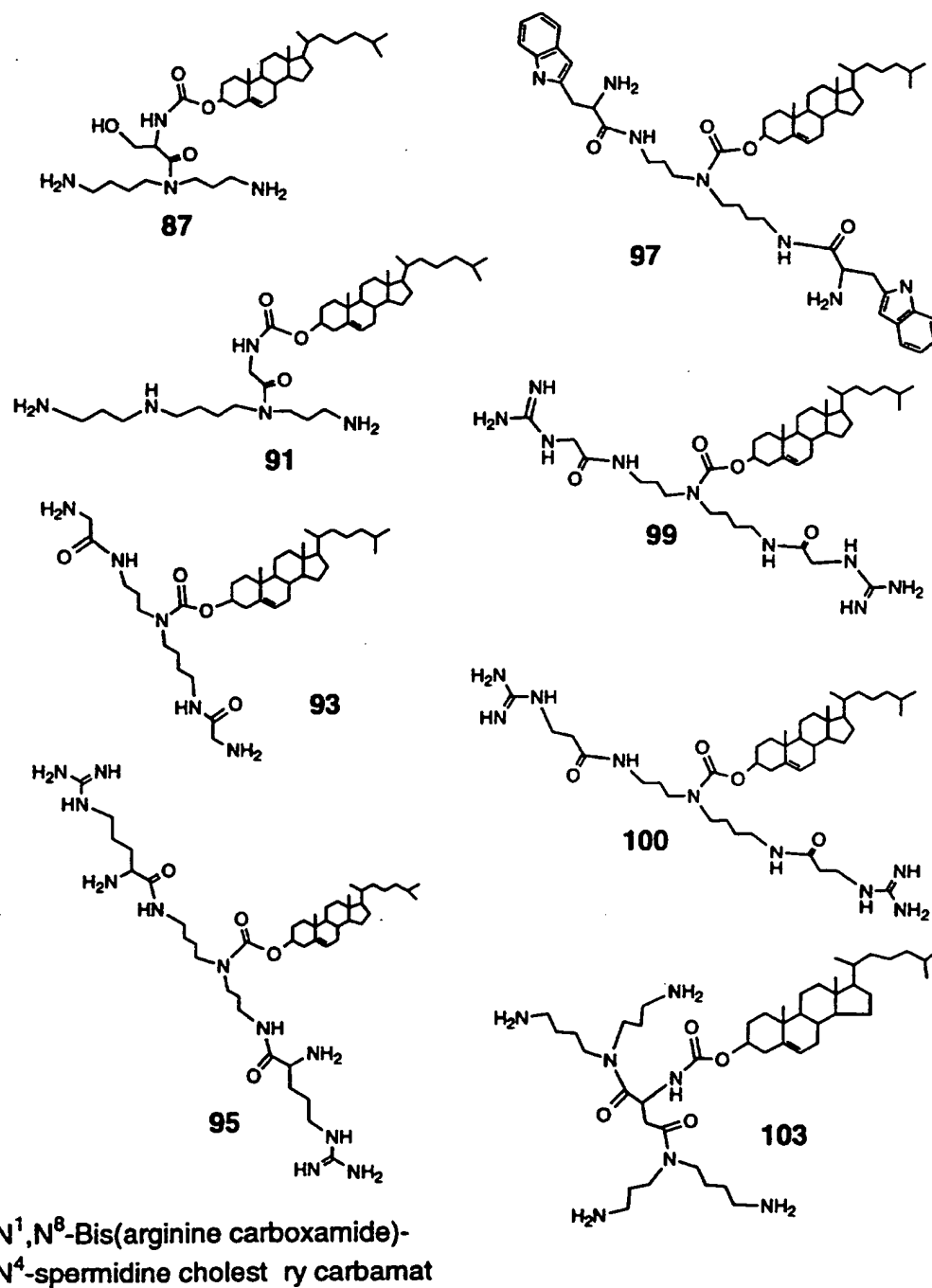
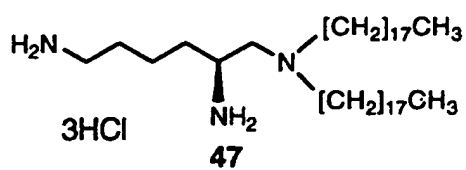
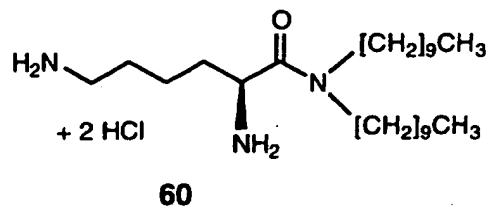
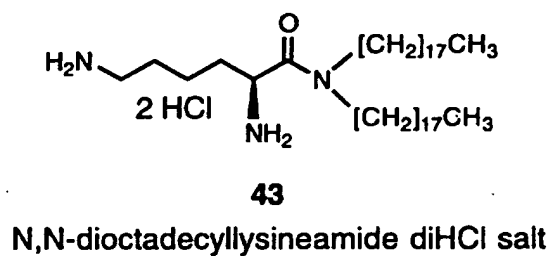
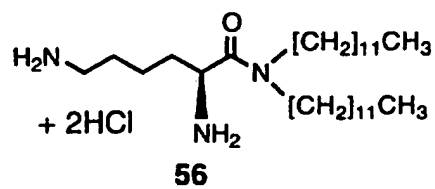
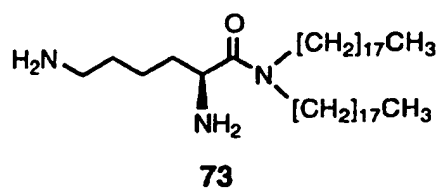


FIG. 5

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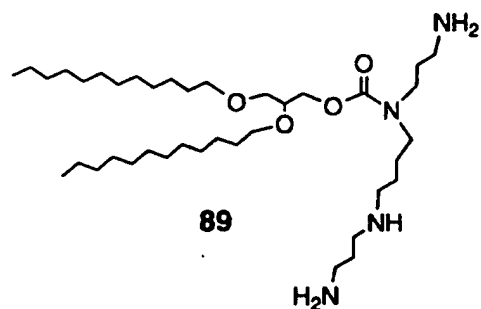
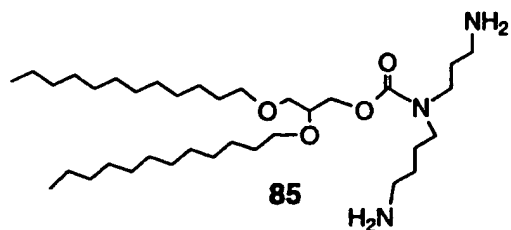
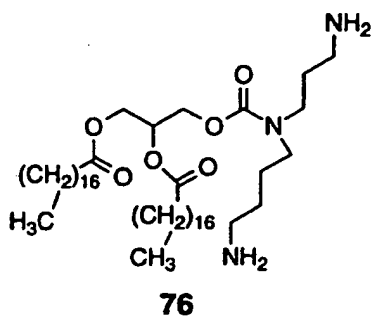
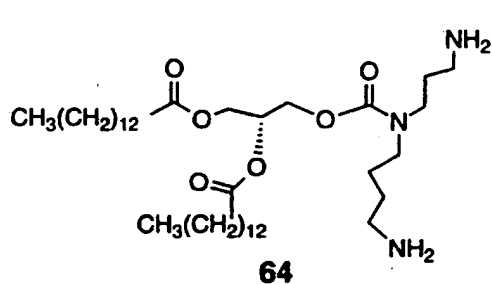


N<sup>1</sup>,N<sup>1</sup>-di-octadecyl-1,2,6-  
triaminohexane tri HCl salt

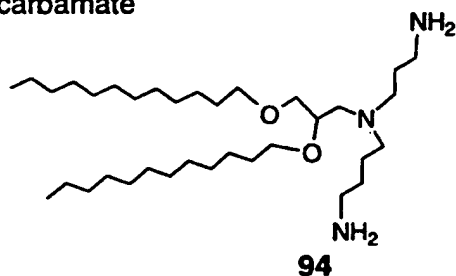


**FIG. 6**

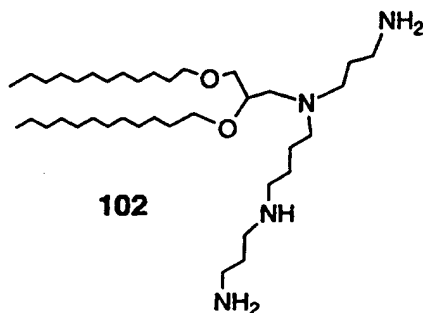
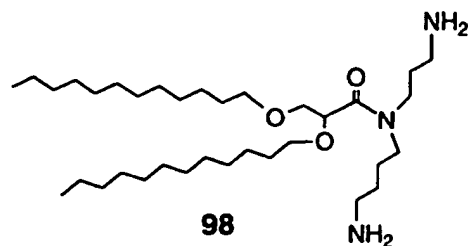
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1-(N<sup>4</sup>-spermine)-2,3-dilaurylglycerol  
carbamate



N<sup>4</sup>-spermidin -2,3-dilauryloxypropylamine



N<sup>4</sup>-spermine-2,3-dilauryloxypropylamine

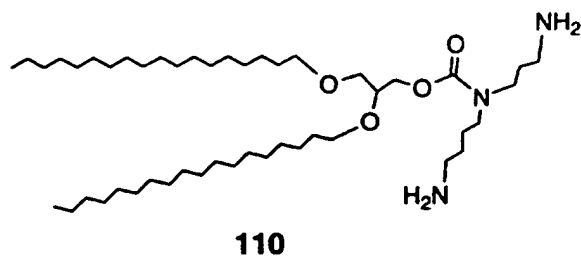
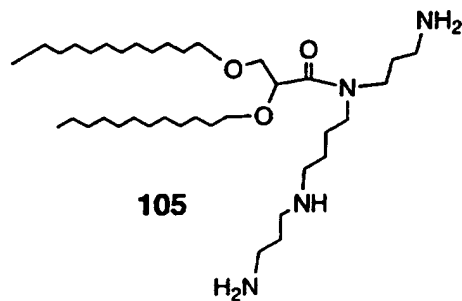


FIG. 7

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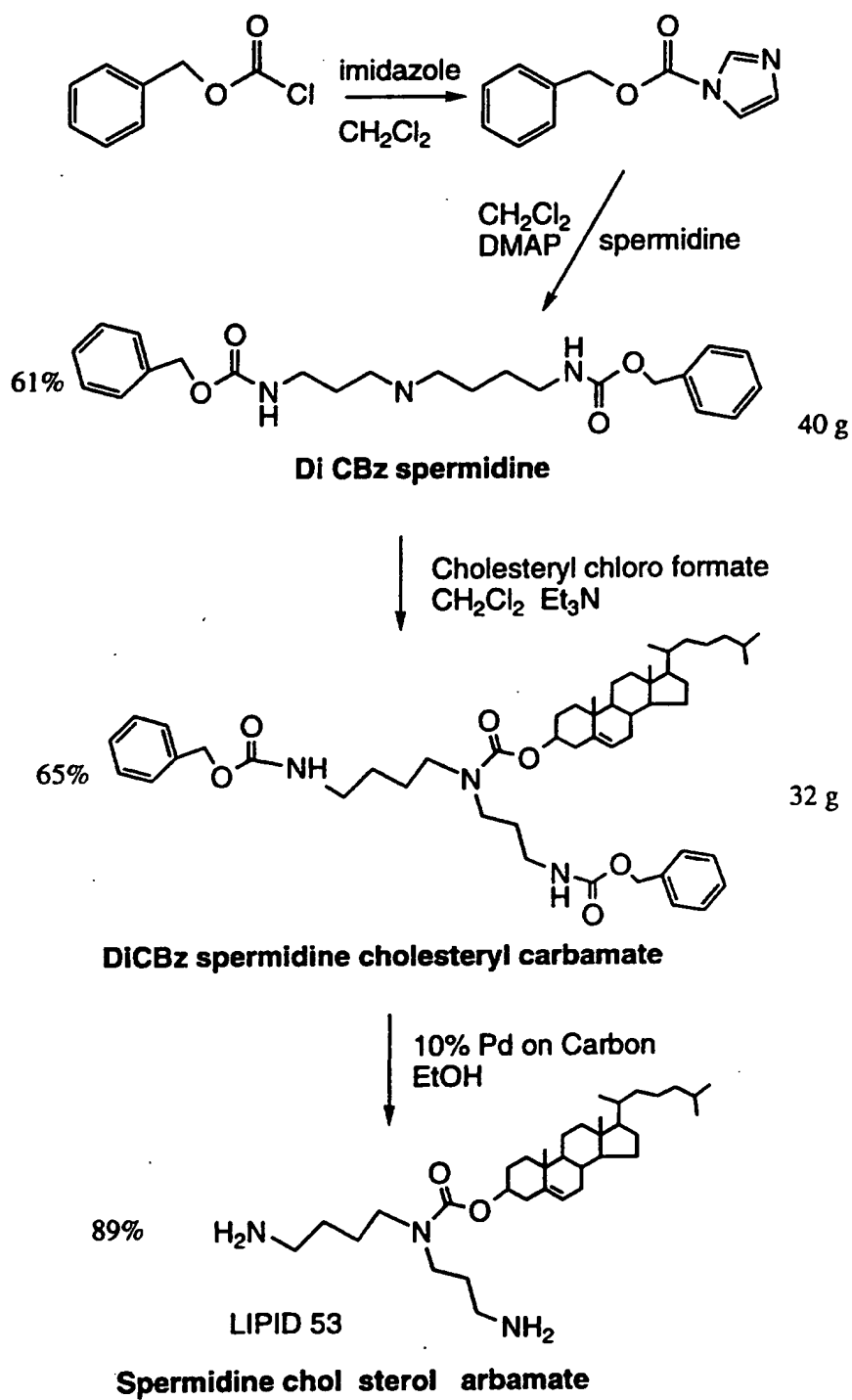


FIG. 8

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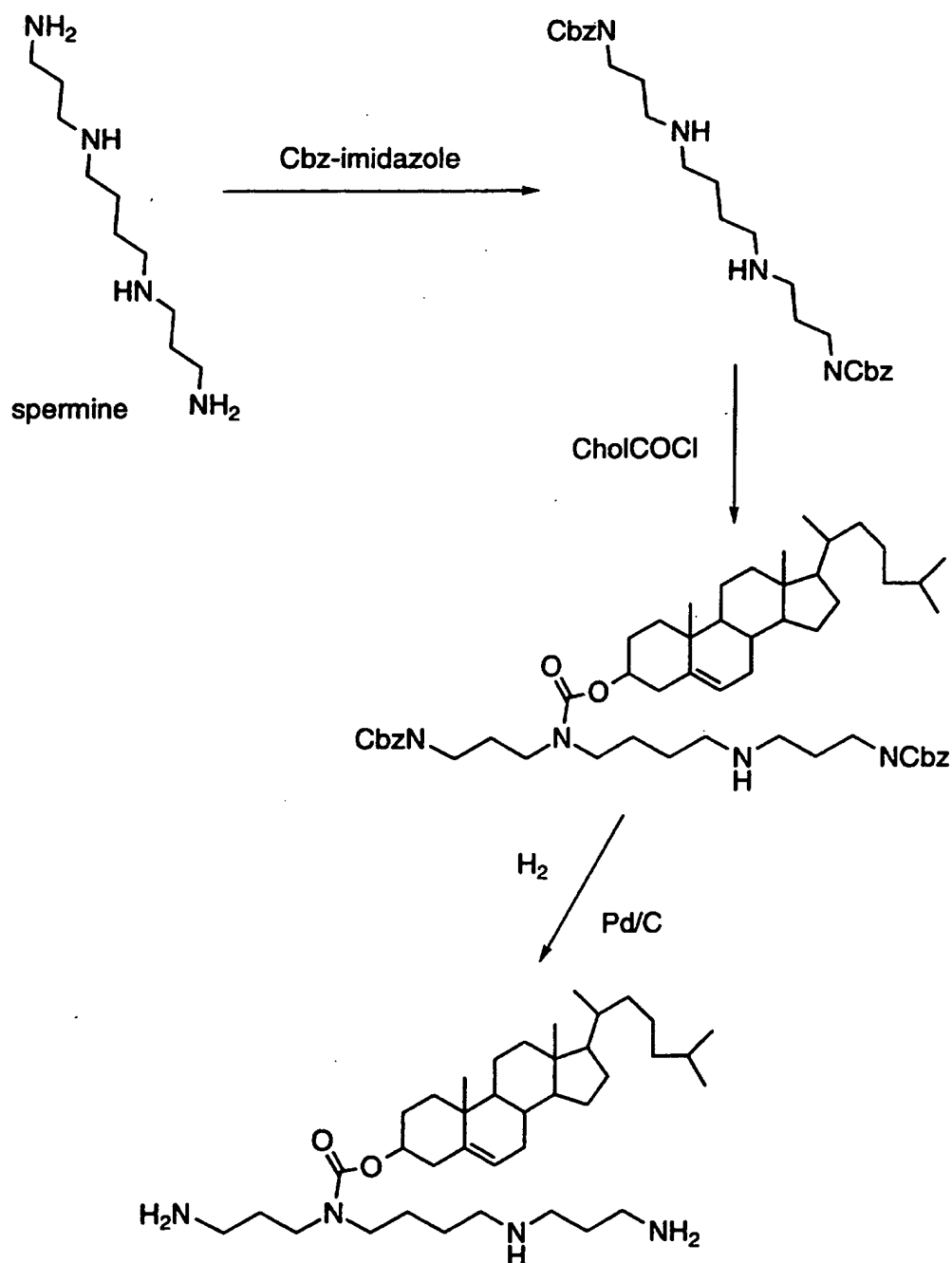


FIG. 9

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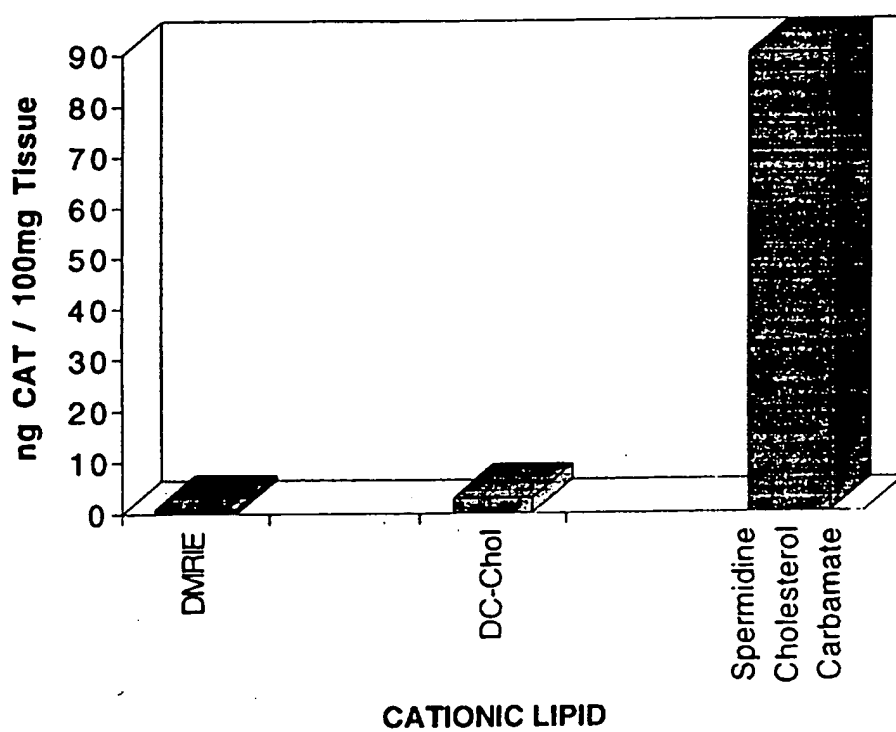


FIG. 10

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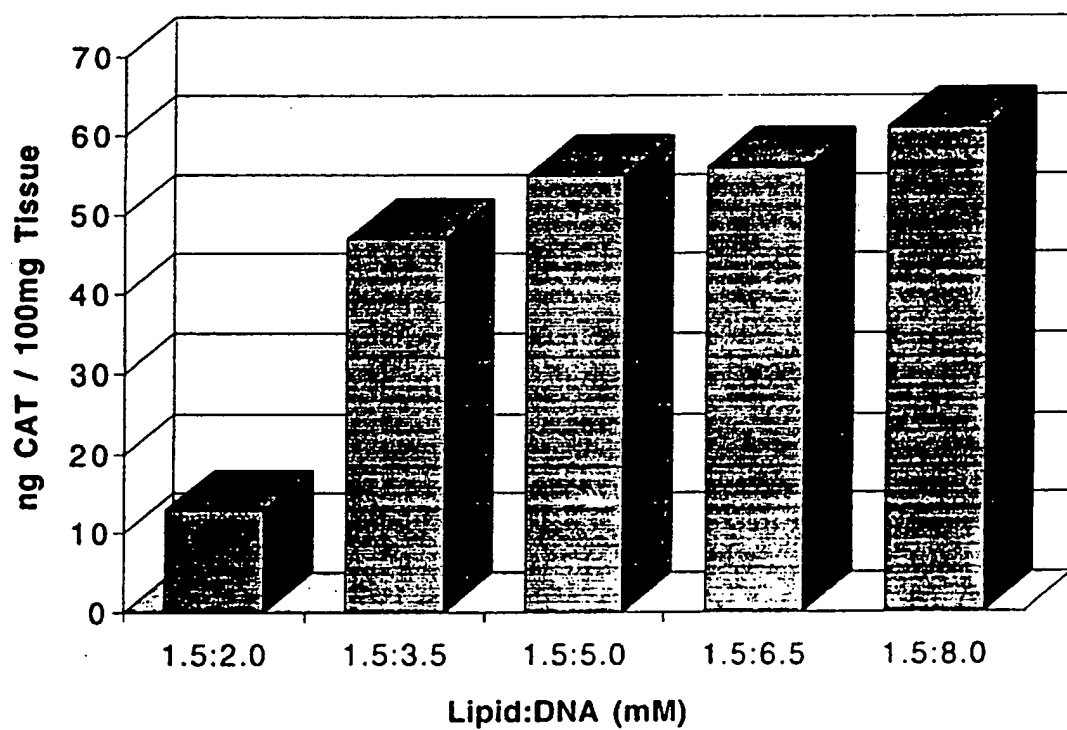


FIG. 11



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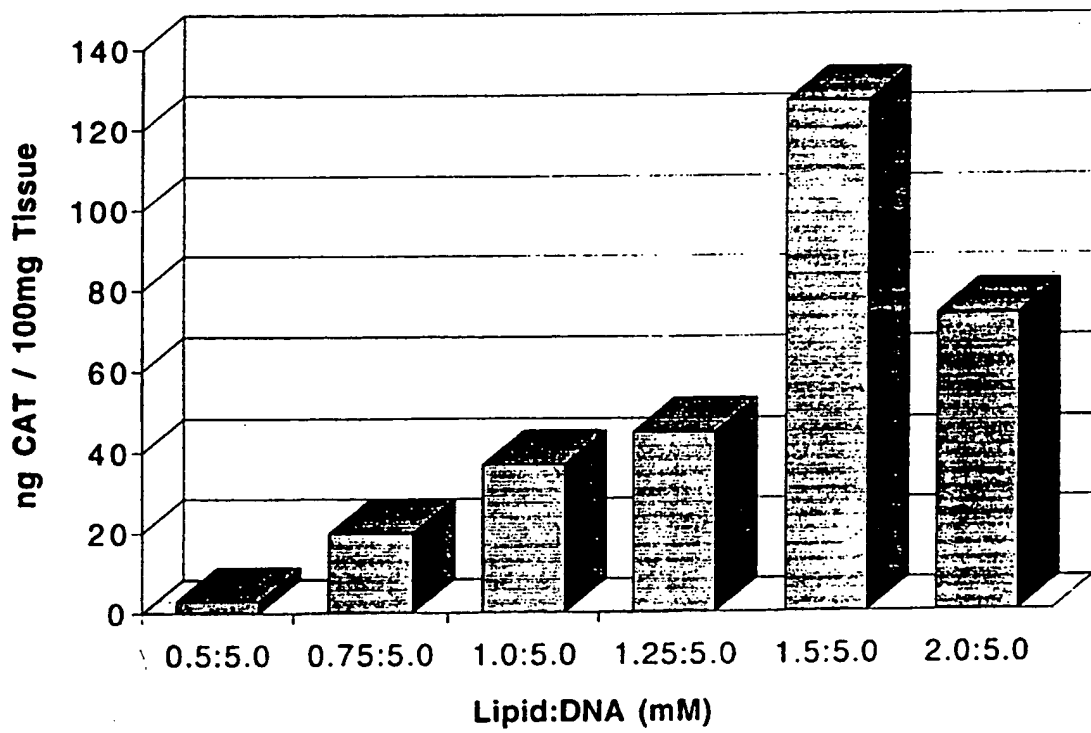


FIG. 12

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<u>Lipid #</u>	<u>Opt. Ratio</u>	<u>In Vitro</u>	<u>In Vivo</u>
53	1\1	4.2	20
65	1\2	2	20
67	1\2	4.8	150
69	2\1	2	2
70	1\1	1	ND
71	1\1	2.4	3
72	1\2	0.7	0.5
75	2\1	5	80
78	1\1	2.3	75
79	1\2	0.8	ND
81	1\2	0.7	ND
82	1\1	3.8	5
83	1\2	4	20
84	1\2	0.4	1
86	1\2	0.5	ND
88			ND
90	1\1	1.8	80
91	1\2	2.1	30
92	1\2	0.5	ND
96	1\2	5	45
101	2\1	1.5	1
104	2\1	0.7	0
106	1\2	1.6	6
107	1\1	1.3	

FIG. 13

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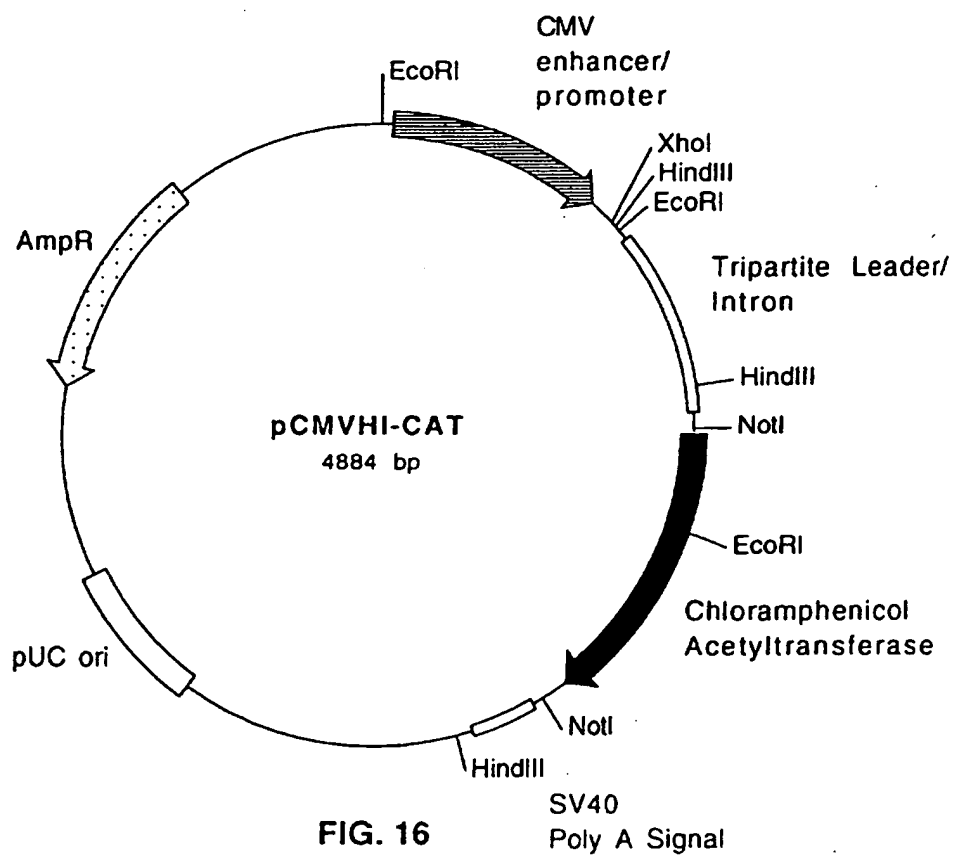
<u>Lipid #</u>	<u>Opt. Ratio</u>	<u>In Vitro</u>	<u>In Vivo</u>
87			
91	1\2	2.1	30
93	1\2	2.7	
95	1\2	3.7	70
97	1\1	0.4	ND
99	1\1	0	ND
100	1\1	0.1	ND
103			

FIG. 14

<u>Lipid #</u>	<u>Opt. Ratio</u>	<u>In Vitro</u>	<u>In Vivo</u>
64	1\1	2.1	1
76	1\1	1	ND
85	1\1	1.5	1
89	1\1	9	15
94	1\1	2	60
98	1\1	1.9	
102	1\2	10	40
105	1\2	3	
110	2\1	0.9	
111			

FIG.15

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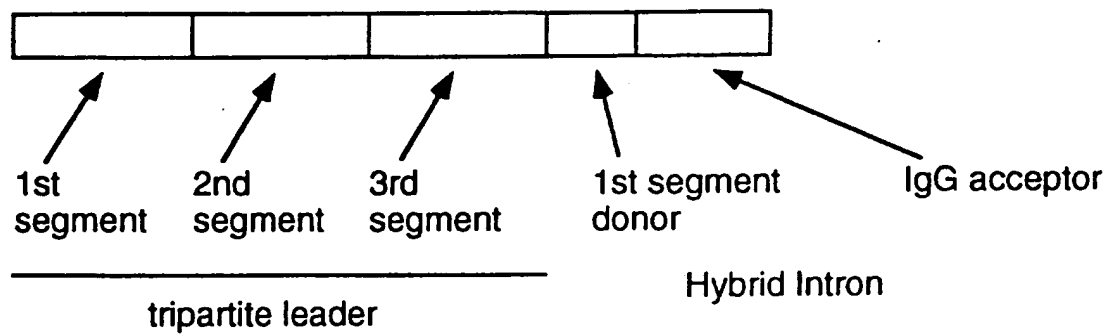


FIG. 17

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FIG. 18A

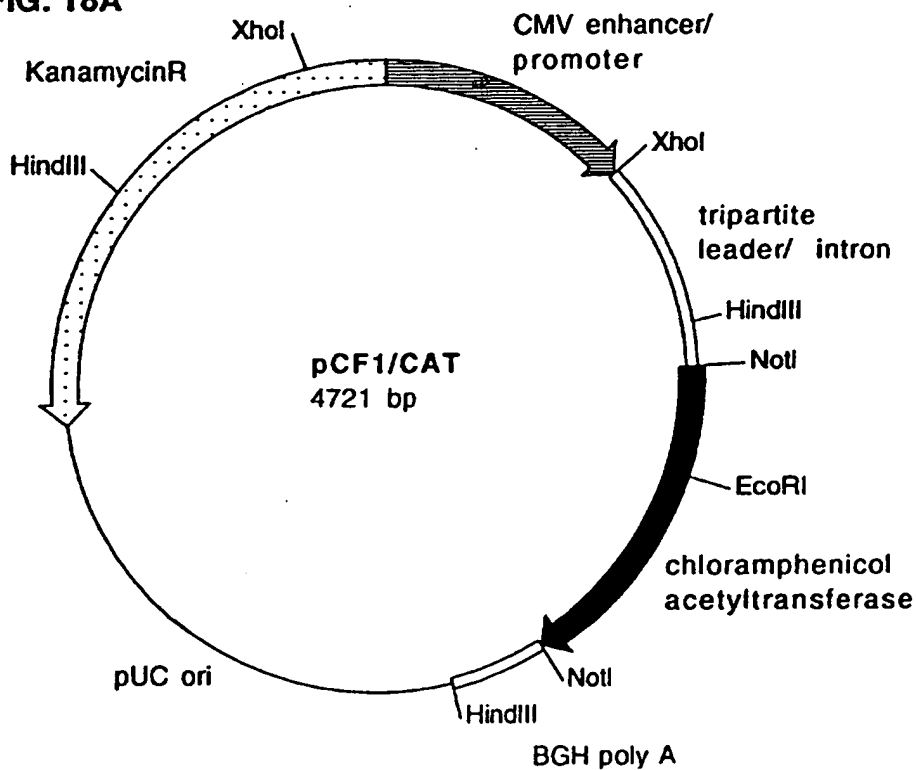
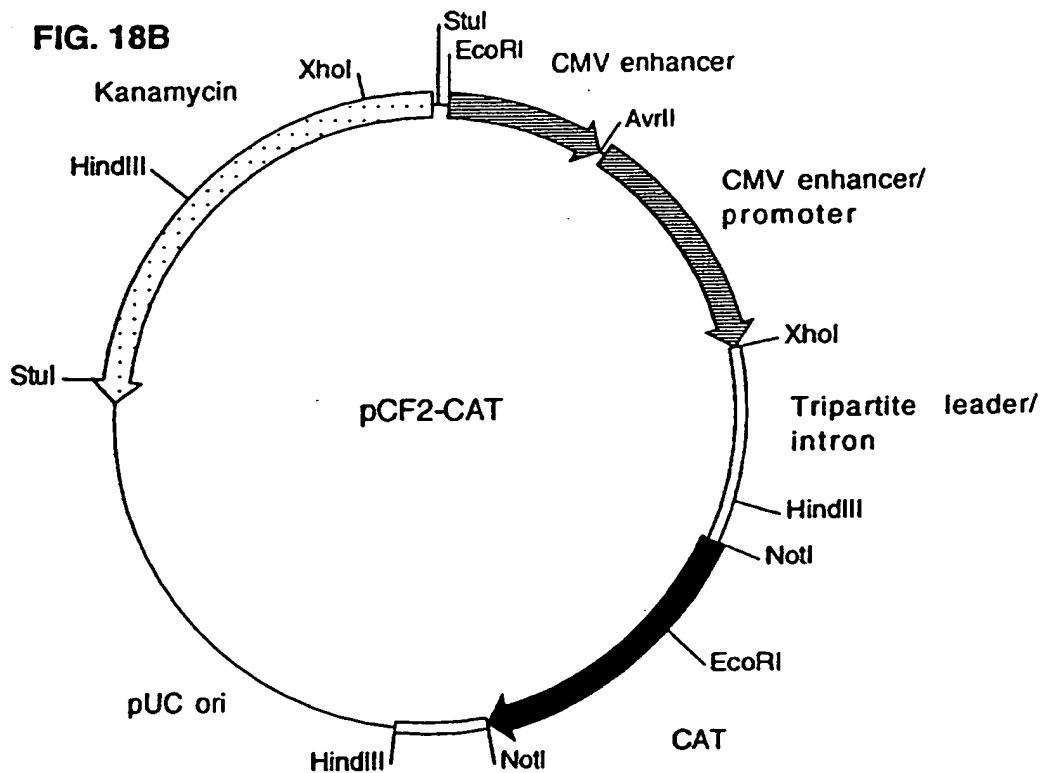
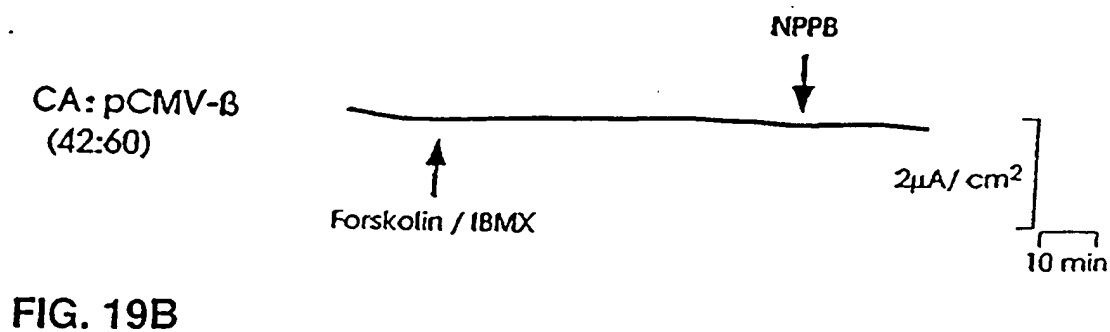
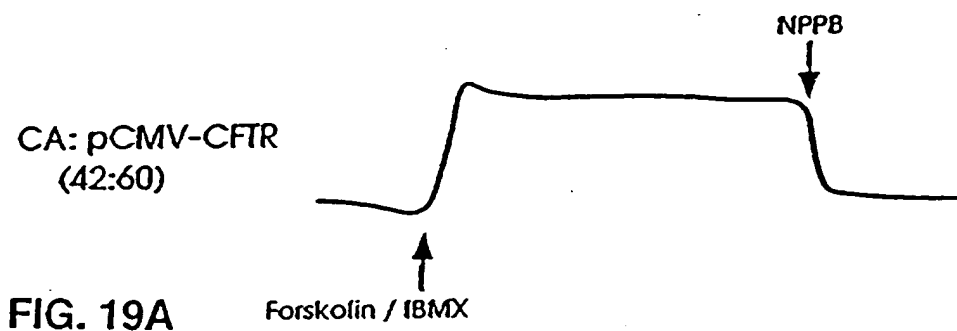


FIG. 18B



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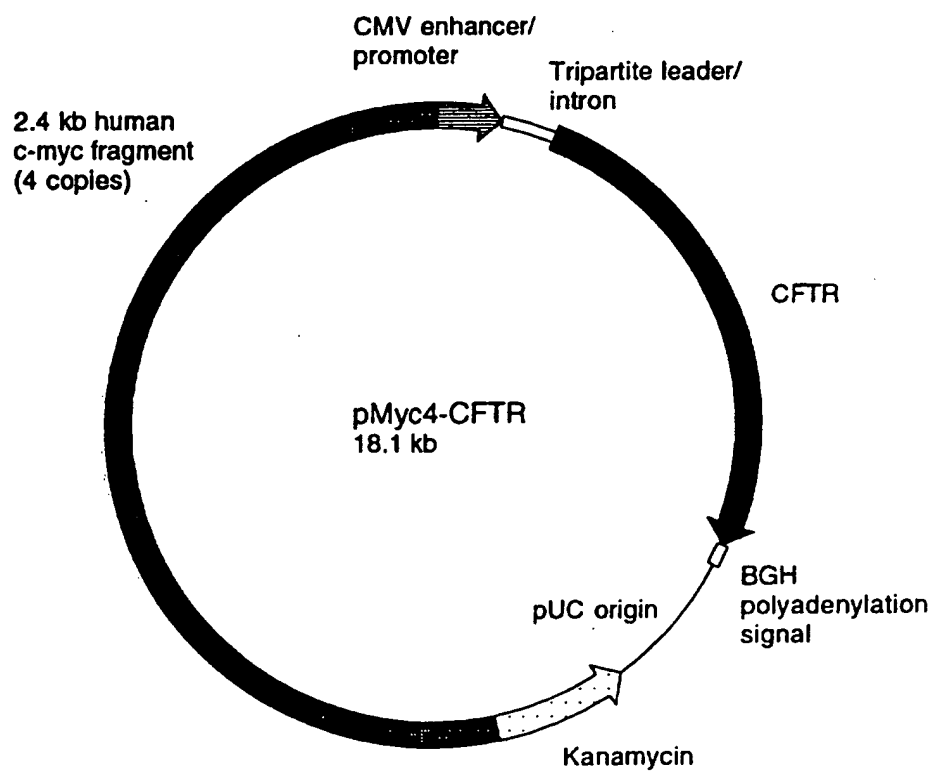


FIG. 20



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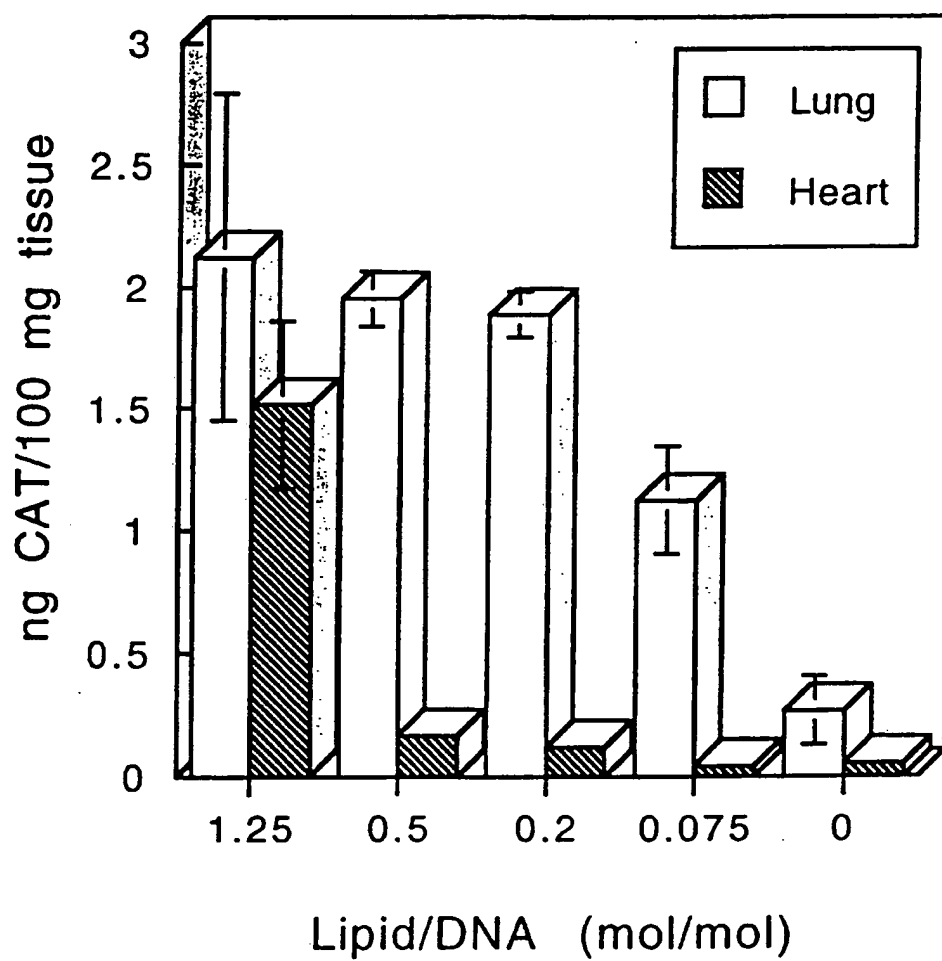


FIG. 21



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification<sup>6</sup> :</b> <b>C07J 41/00, A61K 9/127, 48/00, C12N</b> <b>15/63, C07C 211/13, 237/06, 217/28</b> <b>235/10, 271/20</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 96/18372</b>  <b>(43) International Publication Date:</b> 20 June 1996 (20.06.96)															
<b>(21) International Application Number:</b> PCT/US95/16174  <b>(22) International Filing Date:</b> 8 December 1995 (08.12.95)  <b>(30) Priority Data:</b> <table border="0"> <tr> <td>08/352,479</td> <td>9 December 1994 (09.12.94)</td> <td>US</td> </tr> <tr> <td>60/004,344</td> <td>26 September 1995 (26.09.95)</td> <td>US</td> </tr> <tr> <td>60/004,399</td> <td>27 September 1995 (27.09.95)</td> <td>US</td> </tr> <tr> <td>08/540,867</td> <td>11 October 1995 (11.10.95)</td> <td>US</td> </tr> <tr> <td>08/545,473</td> <td>19 October 1995 (19.10.95)</td> <td>US</td> </tr> </table> <b>(71) Applicant:</b> GENZYME CORPORATION [US/US]; One Kendall Square, Cambridge, MA 02139 (US).  <b>(72) Inventors:</b> SIEGEL, Craig, S.; 15 Bradford Road, Woburn, MA 01801 (US). HARRIS, David, J.; Eastern Avenue, Lexington, MA 02173 (US). LEE, Edward, R.; 175-J Center Street, Lincoln Heights, Quincy, MA 02169 (US). HUBBARD, Shirley, C.; 26 Wiley Road, Belmont, MA 02178 (US). CHENG, Seng, H.; 550 Wall Street, Wellesley, MA 02181 (US). EASTMAN, Simon, J.; 13 Royal Crest Drive, Marlboro, MA 01752 (US). MARSHALL, John; 45 West Walnut Street, Milford, MA 01757 (US). SCHEULE, Ronald, K.; 26 East Street, Hopkinton, MA 01748 (US). YEW, Nelson, S.; 25 Rockdale Hill Circle, West Upton, MA 01568 (US). LANE, Mathieu, B.; 55 Sacramento Street, Cambridge, MA 02138 (US). ROWE, Eric, A.; 196 Sylvan Street, Malden, MA 02148 (US). BAGLEY, Rebecca, G.; 28 Waldon Drive # 17, Natick, MA 01760 (US). CHANG, Chau-Dung; 7 Middleby Road, Lexington, MA 02173 (US).		08/352,479	9 December 1994 (09.12.94)	US	60/004,344	26 September 1995 (26.09.95)	US	60/004,399	27 September 1995 (27.09.95)	US	08/540,867	11 October 1995 (11.10.95)	US	08/545,473	19 October 1995 (19.10.95)	US	<b>(74) Agents:</b> DONAHUE, E., Victor et al.; Genzyme Corporation, One Mountain Road, Framingham, MA 01701-9322 (US).  <b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 6 September 1996 (06.09.96)
08/352,479	9 December 1994 (09.12.94)	US															
60/004,344	26 September 1995 (26.09.95)	US															
60/004,399	27 September 1995 (27.09.95)	US															
08/540,867	11 October 1995 (11.10.95)	US															
08/545,473	19 October 1995 (19.10.95)	US															
<b>(54) Title:</b> CATIONIC AMPHIPHILES AND PLASMIDS FOR INTRACELLULAR DELIVERY OF THERAPEUTIC MOLECULES  <b>(57) Abstract</b>  <p>Novel cationic amphiphiles are provided that facilitate transport of biologically active (therapeutic) molecules into cells. The amphiphiles contain lipophilic groups derived from steroids, from mono or dialkylamines, or from alkyl or acyl groups; and cationic groups, protonatable at physiological pH, derived from amines, alkylamines or polyalkylamines. There are provided also therapeutic compositions prepared typically by contacting a dispersion of one or more cationic amphiphiles with the therapeutic molecules. Therapeutic molecules that can be delivered into cells according to the practice of the invention include DNA, RNA, and polypeptides. Representative uses of the therapeutic compositions of the invention include providing gene therapy, and delivery of antisense polynucleotides or biologically active polypeptides to cells. With respect to therapeutic compositions for gene therapy, the DNA is provided typically in the form of a plasmid for complexing with the cationic amphiphile. Novel and highly effective plasmid constructs are also disclosed, including those that are particularly effective at providing gene therapy for clinical conditions complicated by inflammation. Additionally, targeting of organs for gene therapy by intravenous administration of therapeutic compositions is described.</p>																	

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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 95/16174

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07J41/00 A61K9/127 A61K48/00 C12N15/63 C07C211/13  
C07C237/06 C07C217/28 C07C235/10 C07C271/20

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07J A61K C12N C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,5 283 185 (EPAND RICHARD M ET AL) 1 February 1994 see column 1 - column 2; figures 1,2; example XIII	1-4,7-16
X	WO,A,94 05624 (LIFE TECHNOLOGIES INC) 17 March 1994 see page 6 - page 8	1,2,6-16
X	EP,A,0 490 379 (BERLIN CHEMIE AG) 17 June 1992 see page 4	1,2,5
X	US,A,4 971 803 (LI MING P ET AL) 20 November 1990 see column 2 - column 3	1-4,7-16
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

10 July 1996

Date of mailing of the international search report

22.07.96

Name and mailing address of the ISA

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Moreno, C

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/16174

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	WO,A,88 00824 (LIPOSOME TECHNOLOGY INC) 11 February 1988 see page 44; example III ---	1-4,7-16
X	WO,A,94 20520 (MAGAININ PHARMA ; FRYE LEAH L (US); ZASLOFF MICHAEL A (US); KINNEY) 15 September 1994 A see the whole document	1-4 7,9,11, 16
X	US,A,5 004 737 (KIM YOUNG D ET AL) 2 April 1991 see the whole document ---	1-4
X	BIOCHIMICA ET BIOPHYSICA ACTA. BIOMEMBRANES, vol. 814, no. 2, 11 April 1985, pages 256-264, XP000573084 K. R. PATEL ET AL: "Modification of vesicle surfaces with amphiphilic sterols. Effect on permeability and in vivo tissue distribution." A see the whole document	1-3,7 9,10,16
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, no. 4, February 1993, WASHINGTON US, pages 1354-1358, XP000572356 K. S. MOORE ET AL: "Squalamine: an aminosterol antibiotic from the shark." A see the whole document	1-4 7,9,11, 16
	-----	

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 95/ 16174

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 14-16  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 14-16 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 95/16174

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5283185	01-02-94	AU-B- 665029 AU-B- 2656592 CA-A- 2116676 EP-A- 0663013 JP-T- 7500963 WO-A- 9305162	14-12-95 05-04-93 18-03-93 19-07-95 02-02-95 18-03-93
WO-A-9405624	17-03-94	US-A- 5334761 EP-A- 0656883	02-08-94 14-06-95
EP-A-0490379	17-06-92	DE-A- 4040184 DE-A- 4119235 CA-A- 2057601 JP-A- 5221948	17-06-92 17-12-92 14-06-92 31-08-93
US-A-4971803	20-11-90	NONE	
WO-A-8800824	11-02-88	US-A- 4839175 US-A- 4804539 EP-A- 0316345 JP-T- 2500360	13-06-89 14-02-89 24-05-89 08-02-90
WO-A-9420520	15-09-94	AU-B- 6397494 CA-A- 2157594 EP-A- 0688333	26-09-94 15-09-94 27-12-95
US-A-5004737	02-04-91	NONE	